Niche adaptation and microdiversity among populations of planktonic bacteria

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To my beloved husband Christian

Contents

Chapter 1

Summary

The water column of marine and freshwater habitats might appear as a homogeneous space without visible constraints. However, free-living bacteria struggle for survival and adapted to various ecological niches within this seemingly homogeneous habitat. To understand the processes of niche formation and bacterial speciation, microdiverse clusters of freshwater *Alphaproteobacteria* with identical 16S rRNA gene sequences were investigated. The family *Sphingomonadaceae* was found to be most abundant within the alphaproteobacterial community in the analyzed Walchensee, as determined by a 16S rRNA gene clone library constructed from a four-season mixture of water samples. Two phylotypes, G1A and G7A, dominated the *Sphingomonadaceae* community and both phylotypes were found to be physiologically active *in situ* throughout the year. *Sphingomonadaceae* members were cultivated in low nutrient strength liquid media from water samples obtained from the oligotrophic Walchensee and the neighboring mesotrophic Starnberger See. In total 54 isolates with identical 16S rRNA gene sequences of phylotype G1A and seven isolates of the G7A phylotype were obtained in pure culture.

Based on phylogenetic analysis, phylotype G1A formed a novel genus with less than 95.2% of 16S rRNA gene sequence similarity to established *Sphingomonadaceae*, whereas phylotype G7A was identical to *Sandarakinorhabdus limnophila* DSM 17366^T. Because of the high number of isolates, further studies focused on G1A. One of these isolates, strain G1A_585^T, was analyzed in detail and was proposed as a new genus by the name of *Sphingorhabdus planktonica* sp. nov. (DSM 25081^T), based on the phylogenetically isolated position compared to the closest related genus *Sphingopyxis*. Furthermore strain G1A_585^T exhibited a novel composition of fatty acids, was non motile and harbored an exceptional low G+C content compared to valid *Sphingopyxis* species.

Because all isolates and environmental sequences of G1A harbored identical 16S rRNA gene sequences, different subpopulations were identified using the more variable internal transcribed spacer regions (ITS1) as a marker sequence. Analyses of ITS1 sequences revealed the presence of seven different sequence types among cultured representatives and cloned fragments. Illumina sequencing of seasonal samples yielded 8,576 high quality ITS1 sequences that included 15 major and numerous rare sequence types. The major ITS1-types

followed distinct temporal patterns, suggesting that the corresponding *Sphingomonadaceae* may occupy different ecological niches. To identify potential ecological niches based on substrate utilization within and between the different ITS1-types of cultivated G1A strains a BiOLOG assay was employed. Since the variability of the substrate utilization pattern could be even more variable within an ITS1 type than between different ITS1 types, the potential mechanism of niche separation in *Sphingomonadaceae* cannot be explained by substrate utilization alone and may be related to other traits that have to be elucidated in future experiments.

In addition to experiments targeting the diversity among and within populations of freshwater *Sphingomonadaceae,* a homogeneous bacterial population consisting of members with identical 16S rRNA- and ITS1 sequences was analyzed. The largest known uniform population is formed by low-light adapted green sulfur bacteria within the oxic-anoxic transition zone (chemocline) of the Black Sea. The main objective was to determine the factors that cause the homogeny of the *Chlorobium* BS-1 population, which is in contrast to the microdiversity found among freshwater *Sphingomonadaceae* (phylotype G1A). BS-1 is an obligate anoxygenic phototroph dwelling in 82-110 m below the surface at the center and in 150-180 m water depth at the periphery of the Black Sea. This pattern is caused by the dome-shape of the oxic-anoxic transition zone of the Black Sea. As a consequence of the deep chemocline, *Chlorobium* BS-1 is adapted to the lowest light intensities described thus far.

To restrict the ecological niche of *Chlorobium* BS-1 in the Black Sea the physiological status of *Chlorobium* BS-1 cells at the shallower central positions of the chemocline as well as at the deeper periphery were investigated by measuring the concentrations of the ITS1 transcripts. ITS-RNA of *Chlorobium* BS-1 could only be detected in the shallower central parts of the Black Sea; indicating inactive BS-1 cells at the chemocline of the periphery, were only ITS-DNA was detected. These results are congruent with $H^{14}CO_3^-$ incorporation experiments and bacteriochlorophyll *e* measurements within the same sampling of Evelyn Marschall and Jörg Overmann, who demonstrated that photosynthetic activity of BS-1 during the summer is most likely limited to the central part of the Black Sea with its sallower chemocline, while light intensities observed in the periphery were below the threshold for phototrophic growth. However, during wintertime, measured light intensities remain below the threshold of photosynthetic growth even at the center of the Black Sea. Thus for dormant BS-1 cells little energy must be sufficient to maintain essential cellular functions until photosynthesis becomes possible again in summer. This hypothesis is supported by my ATP

measurements, that demonstrated a maintenance energy requirement of ~1.6-4.9·10^{-15} kJ·$cell^{-1}$·d^{-1} for *Chlorobium* BS-1, which is the lowest value determined for any living bacterial cell thus far.

Despite energy saving, one further possible reason for adaptation of *Chlorobium* BS-1 to extreme energy conditions could be derived from pigment analyses of the natural samples in this study. *Chlorobium* BS-1 cells from deeper locations of the chemocline contained bacteriochlorophyll *e* homologs with longer aliphatic side chains than light saturated incubated laboratory cultures. The resulting red shift of the absorption maximum has been hypothesized to facilitate the channeling of the excitation energy and the energy transfer efficiency from the light harvesting complexes towards the reaction centre.

In conclusion, the uniform population of *Chlorobium* BS-1 is adapted to a narrow niche, the central chemocline of the Black Sea. In contrast freshwater *Sphingomonadaceae* of the G1A phylotype exhibit microdiversity as determined by ITS-sequence analysis and seems to occupy various ecological niches.

Chapter 2

General Introduction

2.1 Bacterial speciation concepts

How bacterial species evolve and which mechanisms are shaping bacterial diversity is yet not completely understood and debated controversially (Cohan & Perry, 2007; Fraser *et al.*, 2009). Since 1987 prokaryotic species are defined as a group of strains exhibiting at least 70% similarity by DNA-DNA hybridization and sharing a substantial proportion of phenotypic characteristics, which distinguish them from other species (Wayne, 1987). This definition has been successfully employed for species identification, but provides only limited insights into evolutionary forces such as mutation, recombination, genetic drift or selection, which shape bacterial diversity (Fraser *et al.*, 2007; Fraser *et al.*, 2009). After the availability of sequencing technology the bacterial species definition was re-assessed and 16S rRNA gene phylogeny, comparison of housekeeping gene sequences or entire genomes were included in the definition (Stackebrandt *et al.*, 2002). However, by studying recent species only a snapshot of bacterial evolution becomes visible, while the process leading to these species also needs to be understood to reveal the underlying evolutionary forces. To understand the process of speciation different models has been developed. The stable ecotype model (Fig. 1A) assumes phylogenetic closely related and ecological distinct populations, so-called ecotypes, coexist within the same habitat (Koeppel *et al.*, 2008). According to the stable ecotype model (Cohan & Perry, 2007; Fraser *et al.*, 2009) periodic selection (selective sweeps) affects a bacterial lineage within a particular ecological niche and eliminates the genetic diversity that was caused by accumulation of neutral mutations over time since the last period of selection (Cohan & Perry, 2007). Consequently, subsequent selective sweeps divide bacterial lineage into distinct ecotypes, since different niches undergo different selective sweeps. Thus, ecotypes form self-contained gene pools and might be considered as the first step of speciation (Fraser *et al.*, 2009). An example for different ecotypes within closely related bacterial strains are the high- and low light adapted *Prochlocococcus* ecotypes of the Atlantic ocean, that differ in less than 3 % of their 16S rRNA gene sequence and are distributed in different depth of the water column (Moore *et al.*, 1998).

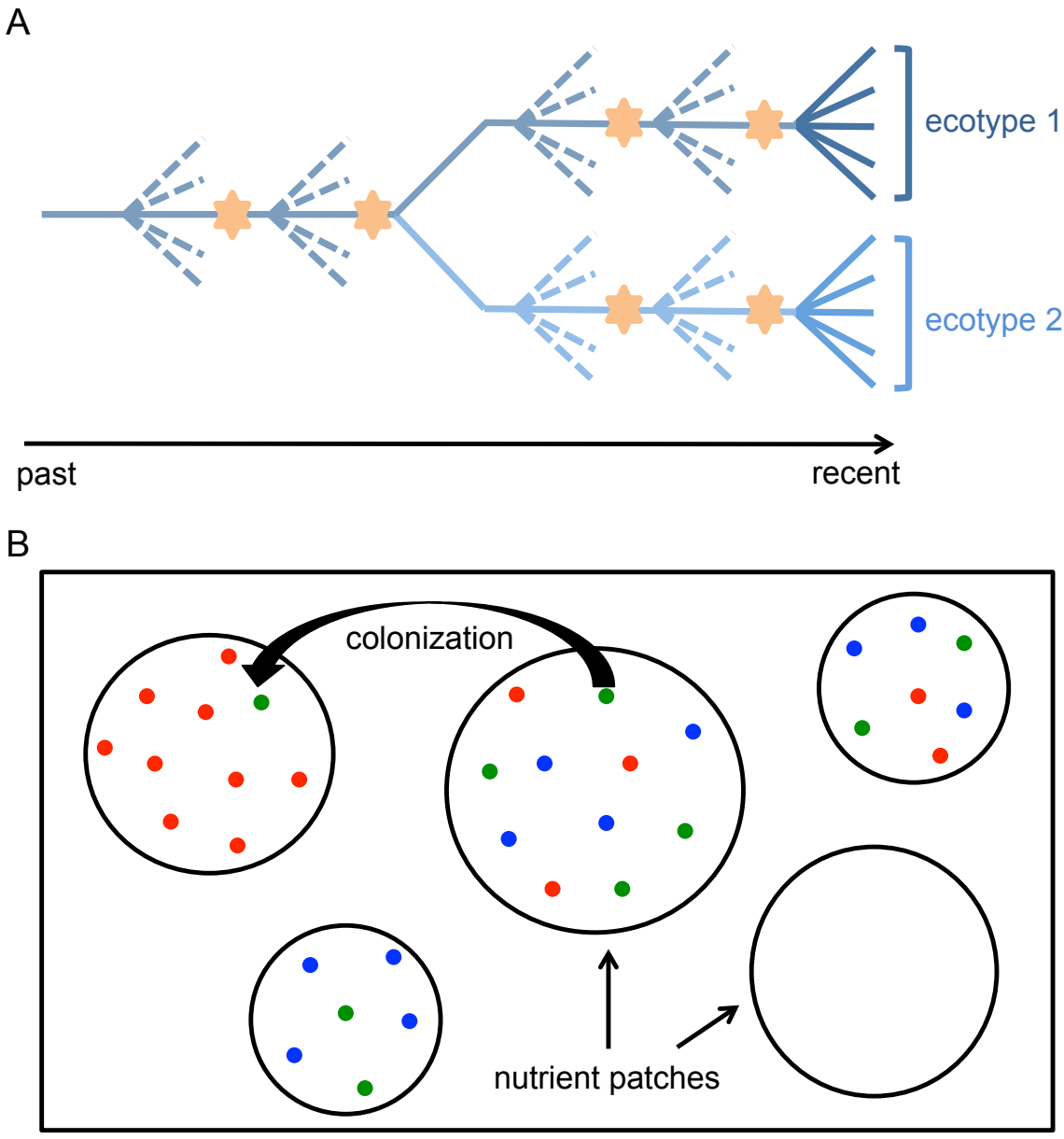

Figure 1A. The stable ecotype model of bacterial speciation. The diagram shows a bacterial lineage that differentiated into two subpopulations (ecotype 1 and ecotype 2) that differ in some aspect of their ecology. At the points marked by asterisks a periodic selection (selective sweep) occurred, that eliminated almost all diversity that has arisen since the last periodic selection. The dashed branches indicating the diversity that was purged by periodic selection and solid branches the existing diversity. When the two populations are ecologically distinct (ecotypes), periodic selection in one population does not influence the diversity in the other and vice versa. Thus each ecotype can become a separate species. (modified after Cohan & Perry, 2007)
B. Theory of metapopulations. Patches (black circles) are not colonized (empty) or may be randomly colonized by a bacterial lineage from another patch (different colors representing distinct lineage). (modified after Fraser *et al.*, 2009)

Another model proposed so-called metapopulations, in which individuals are dispersed between nutrient patches (Keymer *et al.*, 2006) (Fig. 1B). An example are bacterioplankton *Vibrionaceae* strains that were colonizing a wide variety of surfaces including organic particles and zooplankton, while other strains are specialized to a free swimming state and dwell in the water column (Hunt *et al.*, 2008). In contrast to the ecotype model, within a metapopulation the loss of diversity by a selective sweep can be overcome: Strains reintroduced from another nutrient patch can restore diversity. It is likely that in complex environments such as soil or aquatic habitats, a combination of different models is required to explain the development of bacterial diversity (Fraser *et al.*, 2009). Furthermore various different factors that cause genetic diversity such as phage susceptibility (Wolf *et al.*, 2003), horizontal gene transfer (e.g. genomic islands) (Tuanyok *et al.*, 2008) or homologous recombination (Fraser *et al.*, 2007) have to be considered.

2.2 Microdiversity among bacterial populations

More than 50% of the diversity observed in coastal bacterioplankton consists of microdiverse clusters with ≥ 99% 16S rRNA gene sequence identity (Acinas *et al.*, 2004). Similar phenomena could be found for *Bacillus subtilis* and *B. licheniformis* in soils (Connor *et al.*, 2010) or *Firmicutes* and *Bacteroidetes* species in the human intestinal gut (Eckburg *et al.*, 2005). Recently, microdiverse clusters among different freshwater lineages of the betaproteobacterium *Polynucleobacter necessarius,* subspecies *asymbioticus* have been demonstrated. These lineages differ by less than 1% in their 16S rRNA gene sequences, but seems to occupy distinct niches with respect to pH, conductivity, dissolved organic carbon and oxygen concentrations (Jezbera *et al.*, 2011).

In contrast, ecotypes with identical 16S rRNA gene sequences were detected for slope specific populations of *Bacillus simplex* in two canyons (Sikorski & Nevo, 2005). Between isolates of the sun-exposed, south facing slopes and shady, north facing slopes, adaptation to different temperatures were identified as the selective force due to different fatty acid compositions (Sikorski *et al.*, 2008). Since identical 16S rRNA gene sequences might occur among different ecotypes, as observed in the genus *Bacillus*, a more variable genetic marker was required. The ITS1 (internal transcribed spacer) sequence, which is located between the 16S rRNA gene and the 23S rRNA gene, fulfilled this criteria (Barry *et al.*, 1991) and ecotypes could be discriminated by their ITS1 sequence in previous studies (Jaspers & Overmann, 2004; Rocap *et al.*, 2002). For example two *Brevundimonas alba* morphotypes (spirilloid and rod shaped) with identical 16S rRNA sequences could be distinguished by ITS

fingerprints. The rod shaped isolates shared two different ITS types, whereas the spirilloid isolates belonged to a single ITS type (Jaspers & Overmann, 2004). Since population with identical ITS1 sequences could still exhibit different ecotypes (Hahn & Pockl, 2005; Otsuka *et al.*, 1999), ITS-sequence analysis provides first insights regarding the ecotype diversity of a given species.

2.3 Limnic *Sphingomonadaceae* as a model system for bacterial speciation

Sphingomonadaceae represent typical members of freshwater bacterioplankton communities (Gich *et al.*, 2005; Glöckner *et al.*, 2000; Zwart *et al.*, 2002) but also occur widespread in the marine environment, soils and as pathogens (Balkwill *et al.*, 2006; Kim *et al.*, 2007; Takeuchi *et al.*, 2001). Bacteria of this phylogenetic group are physiologically highly diverse (Balkwill *et al.*, 2006). *Sphingomonadaceae* were shown to exhibit diverse physiological traits such as aerobic anoxygenic photosynthesis (Kim *et al.*, 2007). Furthermore typical *Sphingomonadaceae* are aerobic, with exception of different *Zymomonas mobilis* subspecies, that live facultative anaerobe by producing ethanol via fermentation (Kalnenieks, 2006). Many sphingomonads degrade polycyclic aromatic compounds and xenobiotics (Basta *et al.*, 2005) including toxic dioxin pollutants which makes them useful for industrial wastewater treatment (Wittich *et al.*, 1992).

Aquatic *Sphingomonadaceae* representatives were successfully recovered by cultivation on low nutrient media (Page *et al.*, 2004; Pinhassi & Berman, 2003). For example, three isolates of *Sandarakinorhabdus limnophila* with identical 16S rRNA gene sequences were described, that differ within their substrate utilization pattern and growth rates in laboratory cultures (Gich *et al.*, 2005). Thus their abundance, physiological diversity and culturability provided me with the necessary impetus to choose aquatic *Sphingomonadaceae* for the subsequent analysis of bacterial population substructure in this study.

2.4 The impact of environmental conditions on bacterial speciation

While a high diversity among and within populations of different *Sphingomonadaceae* strains was expected, a population of green sulfur bacteria *Chlorobium* BS-1 was described in the Black Sea, that consists of members with identical 16S rRNA- and ITS1 sequences. Thus no microdiversity was observed within this natural population, while the underlying mechanisms remained unknown. The aim of this part of the study was to gain a more solid understanding of the factors, which cause the homogeny of the BS-1 population in the chemocline.

The Black Sea comprises of a 2,000-m-deep sulfidic and anoxic bottom zone that is overlaid with an in average 80-m-thick oxic top layer and a 20-m-thick suboxic intermediate zone (chemocline) (Manske *et al.*, 2005; Murray *et al.*, 1989). The described phylotype *Chlorobium* BS-1 occupies an extraordinary ecological niche in the oxic-anoxic transition zone (chemocline) of the Black Sea, which is located 80-150 m below sea surface. This variation in depth is due to a dual dome shaped structure of the chemocline with cyclonic currents in the centre of the western and eastern basins and several smaller anticyclonic gyres at the periphery (Oguz *et al.*, 1992; Oguz *et al.*, 1993). Consequently the sulfidic zone extends upward to 82-110 m below sea surface in center and remains at 150-180 m at the periphery of the Black Sea (Manske *et al.*, 2005) (Fig. 2). The light intensities in such deep waters corresponds to maximum 0.0009% of the surface light intensities, as measured at the chemocline shallow central positions at solar noon in winter (Manske *et al.*, 2005). While any other phototrophic organism is not able to grow under these low-light conditions, *Chlorobium* BS-1 was shown to be the obligate phototrophic bacterium adapt to the lowest light intensities known so far.

The adaptation of green sulfur bacteria to environments with low light conditions is a general characteristic of this phylum (Overmann & Garcia-Pichel, 2006) and caused by specialized organelle like structures, the chromosomes. They are formed by huge aggregates of light harvesting pigments (bacteriochlorophyll *c, d* or *e*), that are attached to the inner side of the cytoplasmatic membrane (Oostergetel *et al.*, 2010). In addition to the green sulfur bacteria, chlorosomes are also present in some filamentous anoxygenic phototrophs of the phylum *Chloroflexi* and a single aerobic phototroph Candidatus *Chloracidobacterium thermophilum* of the phylum *Acidobacteria* (Bryant *et al.*, 2007).

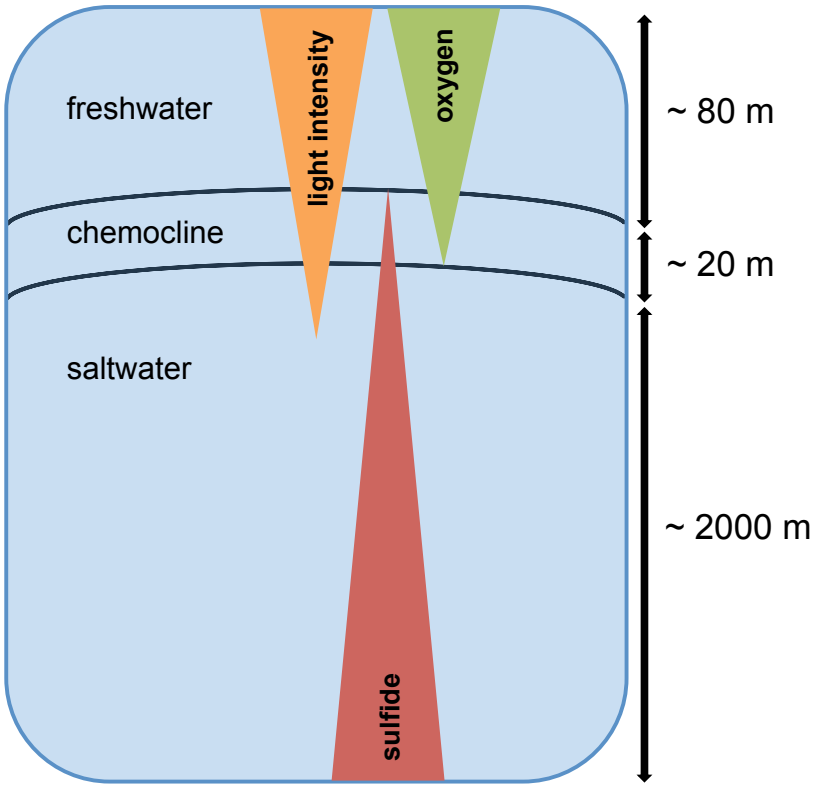

Figure 2. Schematic construction for a section of the Black Sea, containing the relevant factors for *Chlorobium* BS-1 dwelling in the oxic-anoxic transition zone (chemocline). The vertical profiles of sulfide vary strongly across the Black Sea basin and are caused by a dome-shaped structure of the chemocline, whereas the light intensities are comparable within the same depth across the Black Sea. Based on data derived from Manske *et al*., 2005.

2.5 References

Acinas, S. G., Klepac-Ceraj, V., Hunt, D. E., Pharino, C., Ceraj, I., Distel, D. L. & Polz, M. F. (2004). Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* **430**, 551-554.

Balkwill, D. L., Frederickson, J. K. & Fromine, M. F. (2006). *Sphingomonas* and related genera. *Prokaryotes* **7**, 605-629.

Barry, T., Colleran, G., Glennon, M., Dunican, L. K. & Gannon, F. (1991). The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *Genome Res* **1**, 51-56.

Basta, T., Buerger, S. & Stolz, A. (2005). Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. *Microbiol* **151**, 2025-2037.

Bryant, D. A., Costas, A. M., Maresca, J. A., Chew, A. G., Klatt, C. G., Bateson, M. M., Tallon, L. J., Hostetler, J., Nelson, W. C. & other authors (2007). Candidatus *Chloracidobacterium thermophilum*: an aerobic phototrophic Acidobacterium. *Science* **317**, 523-526.

Cohan, F. M. & Perry, E. B. (2007). A systematics for discovering the fundamental units of bacterial diversity. *Curr Biol* **17**, 373-386.

Connor, N., Sikorski, J., Rooney, A. P., Kopac, S., Koeppel, A. F., Burger, A., Cole, S. G., Perry, E. B., Krizanc, D. & other authors (2010). Ecology of speciation in *Bacillus*. *Appl Environ Microbiol* **76**, 1349-1358.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the human intestinal flora. *Science* **308**, 1635-1638.

Fraser, C., Hanage, W. P. & Spratt, B. G. (2007). Recombination and the nature of bacterial speciation. *Science* **315**, 476-480.

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G. & Hanage, W. P. (2009). The bacterial species challenge: making sense of genetic and ecological diversity. *Science* **323**, 741-746.

Gich, F., Schubert, K., Bruns, A., Hoffelner, H. & Overmann, J. (2005). Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. *Appl Environ Microbiol* **71**, 5908-5919.

Glöckner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R. (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. *Appl Environ Microbiol* **66**, 5053-5065.

Hahn, M. W. & Pockl, M. (2005). Ecotypes of planktonic *Actinobacteria* with identical 16S rRNA genes adapted to thermal niches in temperate, subtropical, and tropical freshwater habitats. *Appl Environ Microbiol* **71**, 766-773.

Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J. & Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* **320**, 1081-1085.

Jaspers, E. & Overmann, J. (2004). Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. *Appl Environ Microbiol* **70**, 4831-4839.

Jezbera, J., Jezberová, J., Brandt, U. & Hahn, M. W. (2011). Ubiquity of *Polynucleobacter necessarius* subspecies asymbioticus results from ecological diversification. *Environ Microbiol* **13**, 922-931.

Kalnenieks, U. (2006). Physiology of *Zymomonas mobilis:* some unanswered questions. *Adv Microb Physiol* **51**, 73-117.

Keymer, J. E., Galajda, P., Muldoon, C., Park, S. & Austin, R. H. (2006). Bacterial metapopulations in nanofabricated landscapes. *PNAS* **103**, 17290-17295.

Kim, M. K., Schubert, K., Im, W.-T., Kim, K.-H., Lee, S.-T. & Overmann, J. (2007b). *Sphingomonas kaistensis* sp. nov., a novel alphaproteobacterium containing *pufLM* genes. *Int J Syst Evol Microbiol* **57**, 1527-1534.

Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., Rooney, A. P., Brambilla, E., Connor, N. & other authors (2008). Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *PNAS* **105**, 2504-2509.

Manske, A. K., Glaeser, J., Kuypers, M. M. & Overmann, J. (2005). Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. *Appl Environ Microbiol* **71**, 8049-8060.

Moore, L. R., Rocap, G. & Chisholm, S. W. (1998). Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. *Nature* **393**, 464-467.

Murray, J. W., Jannasch, H. W., Honjo, S., Anderson, R. F., Reeburgh, W. S., Top, Z., Friederich, G. E., Codispoti, L. A. & Izdar, E. (1989). Unexpected changes in the oxic/anoxic interface in the Black Sea. *Nature* **338**, 411-413.

Oguz, T., La Violette, P. E. & Unluata, U. (1992). The Upper Layer Circulation of the Black Sea: Its Variability as Inferred From Hydrographic and Satellite Observations. *J Geophys Res* **97**, 12569-12584.

Oguz, T., Latun, V. S., Latif, M. A., Vladimirov, V. V., Sur, H. I., Markov, A. A. P., E., Korovshchikov, B. B., V., E. V. & Ünlüata, Ü. (1993). Circulation in the surface and intermediate layers of the Black Sea. *Deep-Sea Res I* **40**, 1597-1612.

Oostergetel, G. T., van Amerongen, H. & Boekema, E. J. (2010). The chlorosome: a prototype for efficient light harvesting in photosynthesis. *Photosynthesis research* **104**, 245-255.

Otsuka, S., Suda, S., Li, R., Watanabe, M., Oyaizu, H., Matsumoto, S. & Watanabe, M. M. (1999). Phylogenetic relationships between toxic and non-toxic strains of the genus Microcystis based on 16S to 23S internal transcribed spacer sequence. *FEMS Microbiol Letters* **172**, 15-21.

Overmann, J. & Garcia-Pichel, F. (2006). The phototrophic way of life. In *The Prokaryotes: Ecophysiology and Biochemistry*, pp. 32–85. Edited by M. Dworkin & S. Falkow. New York, USA: Springer.

Page, K. A., Connon, S. A. & Giovannoni, S. J. (2004). Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. *Appl Environ Microbiol* **70**, 6542-6550.

Pinhassi, J. & Berman, T. (2003). Differential growth response of colony-forming α- and γ-*proteobacteria* in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Appl Environ Microbiol* **69**, 199-211.

Rocap, G., Distel, D. L., Waterbury, J. B. & Chisholm, S. W. (2002). Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. *Appl Environl Microbiol* **68**, 1180-1191.

Sikorski, J. & Nevo, E. (2005). Adaptation and incipient sympatric speciation of *Bacillus simplex* under microclimatic contrast at "Evolution Canyons" I and II, Israel. *PNAS* **102**, 15924-11599.

Sikorski, J., Brambilla, E., Kroppenstedt, R. M. & Tindall, B. J. (2008). The temperature-adaptive fatty acid content in *Bacillus simplex* strains from 'Evolution Canyon', Israel. *Microbiol* **154**, 2416-2426.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C. & al., e. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043-1047.

Takeuchi, M., Hamana, K. & Hiraishi, A. (2001). Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *Int J Syst Evol Microbiol* **51**, 1405-1417.

Tuanyok, A., Leadem, B. R., Auerbach, R. K., Beckstrom-Sternberg, S. M., Beckstrom-Sternberg, J. S., Mayo, M., Wuthiekanun, V., Brettin, T. S., Nierman, W. C. & other authors (2008). Genomic islands from five strains of *Burkholderia pseudomallei*. *BMC Genomics* **9**, 566.

Wayne, L., Brenner, D., Colwell, R., Grimont, P., Kandler, O., Krichevsky, M. (1987). Report of the ad hoc commitee on reconciliation of approaches to bacterial systematic. *Intl J Syst Bacteriol* **37**, 463-475.

Wittich, R. M., Wilkes, H., Sinnwell, V., Francke, W. & Fortnagel, P. (1992). Metabolism of dibenzo-p-dioxin by *Sphingomonas sp. strain* RW1. *Appl Environ Microbiol* **58**, 1005-1010.

Wolf, A., Wiese, J., Jost, G. & Witzel, K.-P. (2003). Wide geographic distribution of bacteriophages that lyse the same indigenous freshwater isolate (*Sphingomonas* sp. strain B18). *Appl Environ Microbiol* **69**, 2395-2398.

Zwart, G., Crump, B. C., Kamst-Van Agterveld, M. P., Hagen, F. & Han, S. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**, 141-155.

Chapter 3

Identification and targeted cultivation of abundant novel freshwater sphingomonads and analysis of their population substructure

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3.1 Contribution of the author

The alphaproteobacterial clone library and screening for *Sphingomonadaceae* were designed by Mareike Jogler and performed together with Helge Siemens. Cultivation and isolation of *Sphingomonadaceae* were designed by Mareike Jogler and conducted together with Hong Chen. The DGGE analysis and sequencing of the melting types were performed by Mareike Jogler. The Illumina sequencing approach was established by Mareike Jogler and bioinformatics were performed together with Boyke Bunk. Mareike Jogler and Jörg Overmann written the complete article and provided all Figures and Tables, except figure 6 and supplementary table S4. The BiOLOG assay was performed by Hong Chen and the corresponding figure 6 and supplementary table S4 were provided by Johannes Sikorski as well as the corresponding material/methods and results/discussion sections.

3.2 Abstract

Only little is known with respect to bacterial population structure in freshwater environments. Using complementary culture-based, cloning and high-throughput Illumina sequencing approaches, we investigated microdiverse clusters of bacteria that comprise members with identical or very similar 16S rRNA gene sequences. Two 16S rRNA phylotypes could be recovered by cultivation in low nutrient strength liquid media from two lakes of different trophic status. Both phylotypes were also found to be physiologically active *in situ* throughout most of the year as indicated by the presence of their rRNA sequences in the samples. Analyses of internal transcribed spacer (ITS1) sequences revealed the presence of 7 different sequence types among cultured representatives and the cloned *rrn* fragments. Illumina sequencing yielded 8,576 ITS1 sequences that encompassed 15 major and numerous rare sequence types. The major ITS1-types exhibited distinct temporal patterns suggesting that the corresponding *Sphingomonadaceae* lineages may occupy different ecological niches. However, since strains of the same ITS1-type showed highly variable substrate utilization patterns, the potential mechanism of niche separation in *Sphingomonadaceae* cannot be explained by substrate utilization alone and may be related to other traits.

3.3 Introduction

A prokaryotic species is operationally defined as a phenotypically consistent group of strains exhibiting ≥70% similarity of their genomic DNA and ≥97% sequence identity of the 16S rRNA gene (Rosselló-Mora & Amann, 2001) (≥98.7% according to a more recent suggestion; (Stackebrandt & Ebers, 2006)). Yet, two thirds of the diversity present in coastal bacterioplankton resides in clusters of sequences with ≥99% sequence identity (Acinas *et al.*, 2004), so-called microdiverse clusters. Microdiverse bacterial communities have also been found among, e.g., *Synechococcus* populations in an alkaline siliceous hot spring microbial mat (Melendrez *et al.*, 2011), *Bacillus simplex* populations in soil (Koeppel *et al.*, 2008) and *Firmicutes* and *Bacteroidetes* species in the human distal gut (Eckburg *et al.*, 2005). It has been suggested that such microdiverse clusters within the same species arise by periods of selectively neutral diversification that are punctuated by adaptive mutations and followed by selective sweeps (Cohan, 2002). Consequently, microdiverse clusters may represent populations of bacterial cells that share ecological niches and adaptations and may therefore be regarded as distinct evolutionary entities, so-called ecotypes (Giovannoni, 2004; Majewski & Cohan, 1999). Alternatively, such clusters may also result from neutral evolution alone (Fraser *et al.*, 2007).

Microdiverse clusters of marine *Vibrio splendidus* occur at different water temperatures and hence may represent individual ecotypes (Thompson *et al.*, 2004). Similarly, different ecotypes of *Synechococcus* seem to be adapted to different temperatures in hot spring microbial mats (Melendrez *et al.*, 2011). Ecotypes of the marine oxygenic phototroph *Prochlorococcus* differ by less than 3% sequence divergence of their 16S rRNA genes and partition themselves according to high or low light intensity in the water column of tropical or subtropical oceans (Rocap *et al.*, 2003). Ecotypes of the soil bacteria *Bacillus subtilis* and *B. licheniformis* that typically diverge by 0.3% of 16S rRNA gene sequences are adapted to different soil temperatures and correspondingly exhibit different compositions of temperature relevant fatty acids (Connor *et al.*, 2010). Apart from a study of the marine `*Candidatus* Pelagibacter ubique´ (Vergin *et al.*, 2007), the population structure of typical aquatic oligotrophs is largely unexplored. This is particularly true for freshwater environments. Recently, ecological diversification among different lineages of the betaproteobacterium *Polynucleobacter necessarius* subspecies *asymbioticus* has been demonstrated. These lineages inhabit stagnant freshwaters and differ by less than 1% in their 16S rRNA gene sequences, but seem to occupy distinct niches with respect to pH, conductivity, dissolved organic carbon and oxygen concentrations (Jezbera *et al.*, 2011).

In some oligotrophic freshwater lakes, *Alphaproteobacteria* can account for up to 16% or even 24 % of the detectable *Bacteria* (Alfreider *et al.*, 1996; Glöckner *et al.*, 2000; Salcher *et al.*, 2011). Functional differentiation among strains of the alphaproteobacterial genera *Brevundimonas* or among strains of *Sandarakinorhabdus* with identical 16S rRNA sequences has already been demonstrated (Gich *et al.*, 2005; Jaspers & Overmann, 2004) and may lead to a lasting coexistence of these strains in the natural environment. Sphingomonads represent typical constituents of freshwater bacterioplankton communities (Glöckner *et al.*, 2000; Piccini *et al.*, 2006; Zwart *et al.*, 2002) that can be recovered by cultivation in defined low nutrient liquid media (Gich *et al.*, 2005). Based on the existing information, sphingomonads thus represent a suitable novel target group for studies of bacterial population structure and dynamics in freshwater aquatic habitats.

In the present study we analyzed the population substructure and seasonal dynamics of *Sphingomonadaceae* to gain first insights into the processes that are involved in bacterial speciation and niche formation.

3.4 Material and Methods

3.4.1 Sampling sites and environmental parameters

The oligotrophic alpine Walchensee is located at 802 m a.s.l. and has a maximum depth of 190 m. Samples were collected by boat at a distance of 30 m from the western shore (47°35′N, 11°20′E). Mesotrophic Starnberger See is located 23 km north of Walchensee at 584 m a.s.l. and has a maximum water depth of 128 m. Sampling was done from a pier on the eastern shore near the town of Ammerland (47°54′11N, 11°19′54E). Water samples were collected on December 20, 2007 and on April 28, August 14, and October 23, 2008, at a water depth of 1 m. The pump system employed consisted of an inlet made of two polyvinyl chloride cones that were spaced 1 cm apart and that were connected to a bilge pump via isoversinic tubing (Overmann *et al.*, 1998). Temperature, pH and conductivity of the water samples were determined with a WTW Multi 340i multimeter equipped with a SenTix 41-3 and a TetraCon 325 electrode (WTW, Weilheim, Germany) (Tab. 1).

Table 1. Parameters (pH, Temperature, Conductivity) and total cell counts for the two sampling sites.

Parameter	Walchensee				Starnberger See			
Trophic state[a]	oligotrophic				mesotrophic			
Mixing type[a]	dimictic				monomictic			
Secchi depth (m)	16				6			
NO_3-N ($mg \cdot l^{-1}$)[a]	0.58				0.32			
P-total ($\mu g \cdot l^{-1}$)[a]	< 5.0				9.0			
Chlorophyll a $\mu g \cdot l^{-1}$)[a]	1.5				2.2 - 6.2			
DOC ($mg \cdot l^{-1}$)[a]	1.2				NA			
Sampling date	20.12.2007	28.04.2008	14.08.2008	23.10.2008	20.12.2007	28.04.2008	14.08.2008	23.10.2008
Water temperature (°C)	5.3	7.5	17.7	11.9	4.4	9.4	22.0	13.4
Conductivity ($\mu S \cdot cm^{-1}$)	291	290	266	281	326	324	304	304
pH	8.14	8.2	8.5	8.32	7.95	8.3	8.58	8.4
Total cell counts ($x10^5$ cells$\cdot ml^{-1}$)	4.5 ± 0.5	9.2 ± 1.1	8.9 ± 1.3	7.8 ± 0.9	3.8 ± 0.5	16.0 ± 2.8	9.4 ± 2.1	8.9 ± 1.14

[a] Gich *et al.* 2005

3.4.2 Bacterial cell counts

Water samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and bacterial cells collected onto black polycarbonate filters (pore size 0.1 μm; Millipore GmbH Schwalbach) as described previously (Bruns *et al.*, 2002). Total bacterial cell numbers were determined by epifluorescence microscopy (Zeiss Axiolab microscope equipped with filter set Zeiss Ex 450–490 / FT 510 / LP 515).

3.4.3 Cultivation of planktonic *Sphingomonadaceae*

Water samples from both sampling sites obtained in winter 2007 and summer 2008 were used for cultivation. We used basic synthetic freshwater medium buffered with 10 mM HEPES (Bartscht *et al.*, 1999) and supplemented with 20 canonical amino acids, glucose, pyruvate, citrate, 2-oxoglutarate, succinate (200 μM each), Tween 80 (0.001% v/v) and a fatty acid mixture containing formate, acetate and propionate (200 μM each) (Jaspers *et al.*, 2001). Trace element solution SL 10 (final concentration, 1 $ml \cdot l^{-1}$) and 10-vitamin solution (final concentration, 10 $ml \cdot l^{-1}$) were added. For growth stimulation, the inducers cAMP, *N*-butyryl homoserine lactone, *N*-oxohexanoyl-DL-homoserine lactone and ATP were added at final concentrations of 10 μM each (Bruns *et al.*, 2002).

Aliquots of 200 μl growth medium were dispensed into the wells of sterile 96-well round bottom microtiter plates. Each well was inoculated by employing the multidrop combi apparatus (Thermo electron corporation, Vantaa, Finland), chosing a volume that contained 50 or 200 cells per well (Bruns *et al.*, 2003b). For each microtiter plate 12 wells served as contamination control and hence were left inoculated. Plates were incubated for 6 weeks at 15°C. Bacterial cell growth was determined by turbidity measurement and positive cultures were screened by the *Sphingomonadaceae* specific PCR with primers Sphingo866f and Alf968r. Cultures that tested positive for *Sphingomonadaceae* were streaked on plates containing different combinations of media and gelling agents. These solid media contained basic synthetic freshwater medium and either 1:10 diluted HD medium (consisting of 0.05 % casein peptone, 0.01 % glucose, 0.025 % yeast extract; w/v) or the carbon substrates listed above (amino acids, carbon sources, fatty acids, inducers). Washed agar and gellan gum were used as gelling agents.

The number of culturable cells per well x was calculated from the fraction p of positive wells among all inoculated microtiter wells according to $x = -\ln(1-p)$ (Bruns *et al.*, 2003a). The corresponding 95% confidence interval $CI_{95\%}$ were calculated from p and the total

number of inoculated wells *n* according to

$$CI_{95\%} = \pm 1.96 \cdot \sqrt{\frac{p}{n \cdot (1-p)}}$$

3.4.4 Nucleic acid extraction and cDNA synthesis

For extraction of DNA and RNA, cells from 250 to 500 ml of lake water from Walchensee and Starnberger See were collected onto Isopore polycarbonate membrane filters (pore size 0.1 μm pore, diameter 47 mm; Millipore GmbH, Schwalbach, Germany). DNA was extracted using the protocol of Fuhrman et al. (Fuhrman *et al.*, 1988) as modified by Marschall et al. (Marschall *et al.*, 2010). Concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen, Karlsruhe, Germany) employing a microtiter plate reader (Tecan Infinite M200, Männedorf, Switzerland).

RNA was isolated using a modification of the method of Eichler et al. (Eichler) as described previously (Marschall *et al.*, 2010). Remaining DNA was degraded with DNase I (Fermentas, St. Leon-Roth, Germany) and RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. RNA concentrations were determined with the nanodrop ND-1000 (peglab, Erlangen, Germany). Subsequently, RNA was reverse transcribed into cDNA using the ImProm-II™ Reverse Transcriptase (Promega, Mannheim, Germany) and random hexamer primers according to the instructions of the manufacturer.

3.4.5 Cloning of *rrn*-operon fragments from planktonic *Alphaproteobacteria*

Amplicons of the almost full-length 16S rRNA gene and the first internal transcribed spacer (ITS1) were generated using the alphaproteobacterial specific primer set Alpha19f (5′- CTG GCT CAG ARC GAA CG- 3′; (Manz *et al.*, 1992) and LS48r (5′- ACG TCY TTC ATC GCC T- 3′; (Peplies *et al.*, 2004)). As previously documented (Manz *et al.*, 1992; Peplies *et al.*, 2004), this primer set targets also 16S rRNA and 23S rRNA gene sequences, respectively, of *Deltaproteobacteria* and *Verrucomicrobia*. Therefore, we searched for an improved priming site using all 33,637 *Alphaproteobacteria* 16S rRNA gene sequences and 1,180 *Alphaproteobacteria* 23S rRNA gene sequences available in the SILVA database (http://www.arb-silva.de), employing the probe design tool implemented in the ARB software package (Ludwig *et al.*, 2004). This new analysis did not yield an improved primer, however.

Amplification reactions were performed in a Veriti 96-well Thermal Cycler (Applied Biosystems, Carlsbad, USA) employing the conditions listed in Table 2 and yielded PCR

products with lengths between 2080 and 2130 bp. After purification of PCR products and quantification with PicoGreen (see above), amplification products generated from the four different DNA samples for each lake were mixed at equal portions and then ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturer.

Table 2. Differences in PCR conditions for each amplification protocol used in this study.

Primer[a]	Fragment length	total volume (µl)	$MgCl_2$ concentration (mM)[b]	Annealing temperature and cycles[c]	extension time at 72°C (sec)	BSA (µg·µl^{-1})	DNA Polymerase
Alpha19f LS48r	2080-2130 bp	50	2.25	10 x 67°C 25 x 62°C	90	0.8	1.25 U Taq (Qiagen)
Sphingo866f Alf968r	103 bp	10	1.5	10 x 63°C 25 x 61°C	30	-	0.25 U Taq (Qiagen)
Alpha19f Sphingo866r	847 bp	50	1.5	10 x 67°C 20 x 60°C	60	-	1.25 U Taq (Quiagen)
GC341f Sphingo866r	525 bp	50	2.25	10 x 61°C 20 x 56°C	60	0.8	1.25 U Taq (Quiagen)
G1A-ITS-35f G1A-ITS-595r	560 bp	50	1.5	35 x 52°C	60	-	1.25 U Phusion (Finnzymes)

[a] 1 µM final concentration

[b] The PCR-Buffer contained 1.5 mM

[c] melting temperature was for 30 seconds at 94°C each cycle

3.4.6 Specific detection of *Sphingomonadaceae* 16S rRNA genes

Clones and enrichments containing 16S rRNA genes of *Sphingomonadaceae* were identified by a specific PCR screen developed in the present study. For this purpose, the novel primer Sphingo866f (5´-CGCATTAAGTTATCCGCC-3´) was developed. This primer is specific for the 16S rRNA gene of *Sphingomonadaceae* except the deep branching genus *Sphingosinicella* and *Sphingomonas kaistensis* 16846^T. Primer Sphingo866f was combined with *Alphaproteobacteria* primer Alf968r (5´-GGTAAGGTTCTGCGCGTT-3´; (Neef, 1997) to yield 103 bp-long 16S rRNA gene fragments employing optimized amplification conditions (see Tab. 2).

In order to identify particular *Sphingomonadaceae* phylotypes among the primary enrichment cultures for subsequent isolation trials, larger 16S rRNA gene fragments were required. Therefore, a 846-bp long 16S rRNA gene fragment was generated using primer Alpha19f (see above), the reverse complementary version of primer Sphingo866

(Sphingo866r; 5′-GGCGGATAACTTAATGCG-3′) and the PCR conditions specified in Table 2 (see supplemental material).

3.4.7 Sequencing and phylogenetic analysis

The cloned *rrn*-operon fragments were sequenced by the dideoxynucleotide method on an ABI Prism 3730 genetic analyzer (Applied Biosystems, Carlsbad, USA), employing primers Alpha19f (see above), uni1492r (5′-GGTTACCTTGTTACGACTT-3′; (Lane)), uni1392f 5′-GYACACACCGCCCGT-3′; (Olsen *et al.*, 1986)) and the BigDye v3.1 chemistry. Amplicons obtained from cultures were sequenced directly. Sequences were edited and assembled with the Vector NTI computer package (Invitrogen).

Sequences were screened for the presence of chimeras by the Greengenes software bellerophon and chimeric sequences were removed. Phylogenetic analysis of 16S rRNA gene and ITS1 sequences was conducted with the ARB software package (Ludwig *et al.*, 2004). Sequences were automatically aligned with the integrated Fast Aligner tool of the ARB package, and the alignment was corrected manually according to secondary structure information. Small nucleotide differences ≤0.35 % between 16S rRNA gene or ITS1 sequences are within the error range of the *Taq* polymerase (Ling *et al.*, 1991; Saiki *et al.*, 1988). Consequently, such small nucleotide differences were only considered in the sequence analysis if they were verified for different clones and could be confirmed by secondary structure analysis (i.e., if two single nucleotide exchanges were found at complementary positions within double helix regions of the 16S rRNA). In addition, small nucleotide differences were confirmed by repeating the PCR reaction and sequencing.

The 16S rRNA gene and ITS1 phylogenetic trees were constructed based on Maximum Likelihood, Neighbor Joining and Maximum Parsimony algorithms. Phylogenetic trees were generated with the ARB software package and bootstrap values were calculated with 1,000 bootstrap resamplings. We identified 16S rRNA gene sequences of the closest relatives by the NCBI BLAST online tool (Altschul, 1997) and classification was verified by the RDP classifier (Wang *et al.*, 2007). Rarefaction curves, diversity indexes (Shannon, Simpson) and richness analysis (Chao1, ACE) were calculated with DOTUR (Schloss & Handelsman, 2005).

3.4.8 Phylogenetic fingerprinting by DGGE

Seasonal changes in the composition and in the composition of the active fraction of *Sphingomonadaceae* in Walchensee and Starnberger See were analysed by comparative phylogenetic fingerprinting employing denaturing gradient gel electrophoresis. From the *Sphingomonadaceae* 16S rRNA genes and 16S rRNA-cDNA 525 bp-long fragments were generated employing the bacterial primer GC341f (Muyzer *et al.*, 1993), the specific primer Sphingo866r (see above) and the appropriate cycling conditions (specified in Table 2). Separate amplification products were generated for the DNA and cDNA of each sampling date and each lake.

PCR products were separated by denaturing gradient gel electrophoresis (DGGE) in 6 % (wt/vol) polyacrylamide gels containing a linear gradient of 35 % to 65 % denaturant (Overmann & Tuschak, 1997) using the Ingeny phorU system (Ingeny International BV, Goes, Netherlands). Electrophoresis was performed at 60°C with 200 V for 15 min followed by 16 h at 100 V. Polyacrylamide gels were stained with SYBRGold (MoBiTec, Göttingen, Germany) for 30 min. The generated DGGE profiles were analyzed using the Gel ComparII Package (Applied Maths, Sint-Martens-Latem, Belgium).

Representative DNA bands were excised from the gel with a sterile scalpel, transferred to 25 µl of 10 mM Tris-HCl buffer (pH 8.0) and DNA was extracted from the gel pieces by overnight incubation at 4°C. One µl of the supernatant was used as template in subsequent PCR, employing the corresponding primers without a GC clamp. PCR products were sequenced after cleaning. Three DNA fragments yielded two sequence types that were identified after cloning using a TOPO TA cloning kit (Invitrogen, Darmstadt, Germany). Sequence analysis revealed that the presence of two sequences in the same band had to be attributed to cross-contamination by the high amount of *S. limnophila* 16S rRNA genes in the same lane, rather than to the formation of heteroduplexes. Sequences were classified using BLAST (Altschul, 1997) and the ARB software package.

3.4.9 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in the present study were deposited in the GenBank database under accession numbers JF275006-JF275059, JF297619-JF297643 and JN087937-JN087939.

3.4.10 Population substructure of the G1A and G7A phylotype

In order to investigate the population substructure within the G1A and G7A phylotypes, the ITS1 region located between the 16S rRNA gene and the 23S rRNA genes was analyzed for the natural populations as well as the available isolates. A primer set consisting of G1A-ITS-35f (5´-AAGGATTTCGGCGGAA-3´) and G1A-ITS-595r (5´-CTATTTGATTTGTAAC AGCAC-3´) was devised that permitted a specific amplification of a 560 bp-long ITS1 fragment of bacteria of the G1A phylotype using the PCR conditions listed in Table 2. Separate amplification products were generated for the DNA and cDNA of each sampling date and each lake. The cDNA samples were preamplified for 15 cycles with the described conditions and 1 µl PCR produced was used for the second amplification for 35 cycles. Products were purified via the NucleoSpin extract II Kit gel extraction protocol (Macherey-Nagel, Düren, Germany).

ITS-PCR products were analyzed by paired end sequencing (covering 150 bp from each end, including the variable regions) employing the Illumina Genome Analyzer IIx. Libraries of ITS fragments were prepared with NEBNext DNA Sample Prep Master Mix Set 1 (NEB, Frankfurt am Main, Germany) according to the instructions the manufacturer. The fluorescent images were processed to sequences using the Genome Analyzer Pipeline Analysis software 1.9. (Illumina).

Sequence reads containing the primers for amplicon sequencing were trimmed to a fixed length of 110 because of low sequence quality at the ends of sequence reads and the forward and reverse reads were concatenated. Because small sequence differences had to be detected, sequences were then strongly filtered allowing no Phred quality score below 20 for each nucleotide. Both tasks were performed applying custom Java programs. FastQ files were converted to FASTA and Quality files using Biopython (Cock *et al.*, 2009). Unique sequences were determined applying the unique.seqs command from Mothur (Schloss *et al.*, 2009) after filtering for remaining ambiguous nucleotides. Based on the sequence database generated for each sampling date, seasonal differences in abundance were determined for unique sequence types that represented a fraction of >5% in at least one of the samples.

3.4.11 Physiological testing of G1A isolates

The Gen III microplate (BiOLOG, Hayward, CA, USA) (http://www.biolog.com/GENIIILaunch/GEN_III_Promo_Flier.pdf) tests the utilization of 71 sole carbon source and the effect of 23 inhibitory substances by measuring cell respiration by

reduction of tetrazolium salts. The cells were streaked on agar plates containing 1:10 diluted HD and incubated for 2-7 days to yield sufficient cell mass. Gen III microplates were inoculated with cells resuspended in the inoculation fluid IF-A according to the recommendations of the manufacturer (www.biolog.com). Subsequently, the plates were incubated in the dark at 28°C and the intensity of the red color resulting from the reduction of the tetrazolium salt was measured after 3-6 days using the Omnilog-PM reader in the Single Read ID mode. The maximum intensity value is 400 (Barry Bochner, BiOLOG, personal communication). Prior to further analysis, the value of the negative control A1 well was subtracted from all other wells. In order to determine the variability of individual phenotypic traits for the strains of ITS type 4 (N =16) or the pooled strains of ITS type 2, 3, 5 and 6 (N = 13), the variance values were plotted using the R! package ggplot2 (Wickham, 2009). Boxplots were constructed using the geom_boxplot function in ggplot2.

3.5 Results and Discussion

3.5.1 *Sphingomonadaceae* represent a diverse and dominant group of *Alphaproteobacteria* in Walchensee

A clone library of alphaproteobacterial *rrn* fragments was established employing primers Alpha19f and LS48r that target the 5´-ends of the 16S and 23S rRNA genes, respectively. The resulting amplicons cover the almost entire 16S rRNA gene and the complete first internal transcribed spacer (ITS1) region of the *rrn* operon. In order to cover a larger fraction of the alphaproteobacterial diversity that was present in Walchensee, amplification products from bacterioplankton sampled at the four seasons were pooled at equal amounts before cloning and sequencing. A total of 725 clones were generated from this mixed sample and subsequently analyzed.

In a first step, a limited number of 125 clones were sequenced to determine the relative abundance of different alphaproteobacterial subgroups in the clone library. 66 of the clones were identified as 16S rRNA gene sequences of *Alphaproteobacteria* (Fig. 1 and Supplementary Fig. S1). In addition to alphaproteobacterial sequences, clones affiliated with the phyla *Deltaproteobacteria* and *Verrucomicrobia* were present in the library, reflecting the established fact that primer Alpha19f is not fully specific for the 16S rRNA gene sequence and LS48r for the 23S rRNA gene sequence of *Alphaproteobacteria* (Manz *et al.*, 1992; Peplies *et al.*, 2004). The sequences of *Alphaproteobacteria* were phylogenetically diverse and affiliated with 8 families and 5 subgroups of uncultured members (Fig. S1 in the supplementary material). The results for the Walchensee bacterioplankton community are therefore in line with those of previous studies that demonstrated a high diversity of bacteria of this subphylum in other freshwater lakes (Ettema & Andersson, 2009; Glöckner *et al.*, 2000; Zwart *et al.*, 2002). In the Walchensee clone library, *Sphingomonadaceae* comprised 27% of the cloned sequences and hence constituted the dominant group of planktonic *Alphaproteobacteria* (Fig. 1). By comparison, *Caulobacteraceae* (12%), *Hyphomonadaceae* (11%) and *Acetobacteraceae* (11%) were less abundant in the clone library.

Sphingomonadaceae represent typical members of freshwater bacterioplankton communities (Gich *et al.*, 2005; Glöckner *et al.*, 2000; Zwart *et al.*, 2002) but also occur widespread in the marine environment, in pristine and contaminated soils, the rhizosphere, clinical specimen as well as deep subsurface aquifers and sewage treatment plants (Balkwill *et al.*, 2006; Kim *et al.*, 2007; Takeuchi *et al.*, 2001). Bacteria of this phylogenetic group are physiologically highly diverse (Balkwill *et al.*, 2006). Furthermore, aquatic *Sphingomonadaceae*, including some oligotrophic representatives, have been successfully

recovered by cultivation on low nutrient media (Page *et al.*, 2004; Pinhassi & Berman, 2003). Because of their abundance, physiological diversity and culturability, *Sphingomonadaceae* were chosen as the target group for the subsequent analysis of bacterial population substructure.

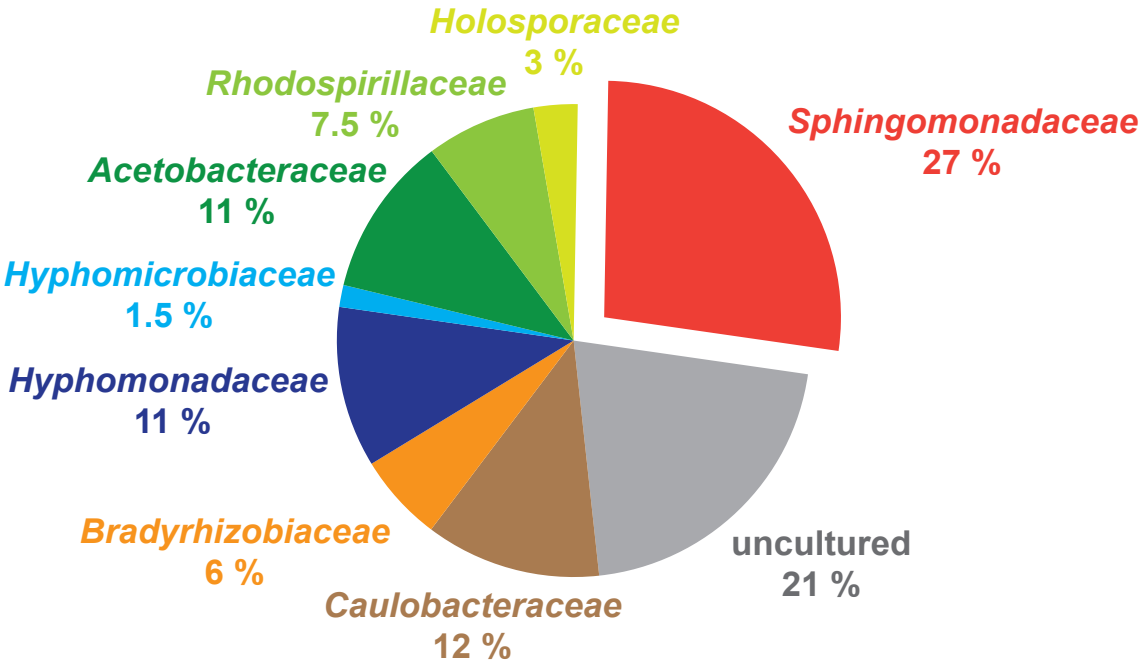

Figure 1. Fractions of 16S rRNA gene sequences within the clone library of Walchensee bacterioplankton affiliated with the different families of *Alphaproteobacteria*.

In order to identify all available *Sphingomonadaceae* clones, a specific PCR protocol was developed and used to screen the 725 clones of the alphaproteobacterial 16S rRNA gene library that had been established for Walchensee bacterioplankton. This yielded a total of 80 clones of the bacterial target group. All PCR-positive clones represented 16S rRNA gene sequences of *Sphingomonadaceae*, confirming the high specificity of our new PCR protocol. A detailed phylogenetic analysis placed the 80 sequences in 11 separate phylotypes (G1A, G2B, G2D, G4A, G5B, G6B, G7A, G7B, G7C, G7D, G8A; Fig. 2 A, B). Six of these phylotypes consisted of more than 3 clones with 100 % sequence identity. The two phylotypes G1A and G7A (Fig. 2A, shaded in grey) by far dominated the clone library and comprised 20 (25 %) and 34 clones (42.5 % of the clones), respectively (Fig. 2B, black columns). Based on our phylogenetic analysis, phylotype G1A forms a novel genus with ≤ 95 % 16S rRNA gene sequence similarity to established *Sphingomonadaceae*, whereas phylotype G7A is identical to *Sandarakinorhabdus limnophila* DSM 17366^T that was previously isolated from Walchensee.

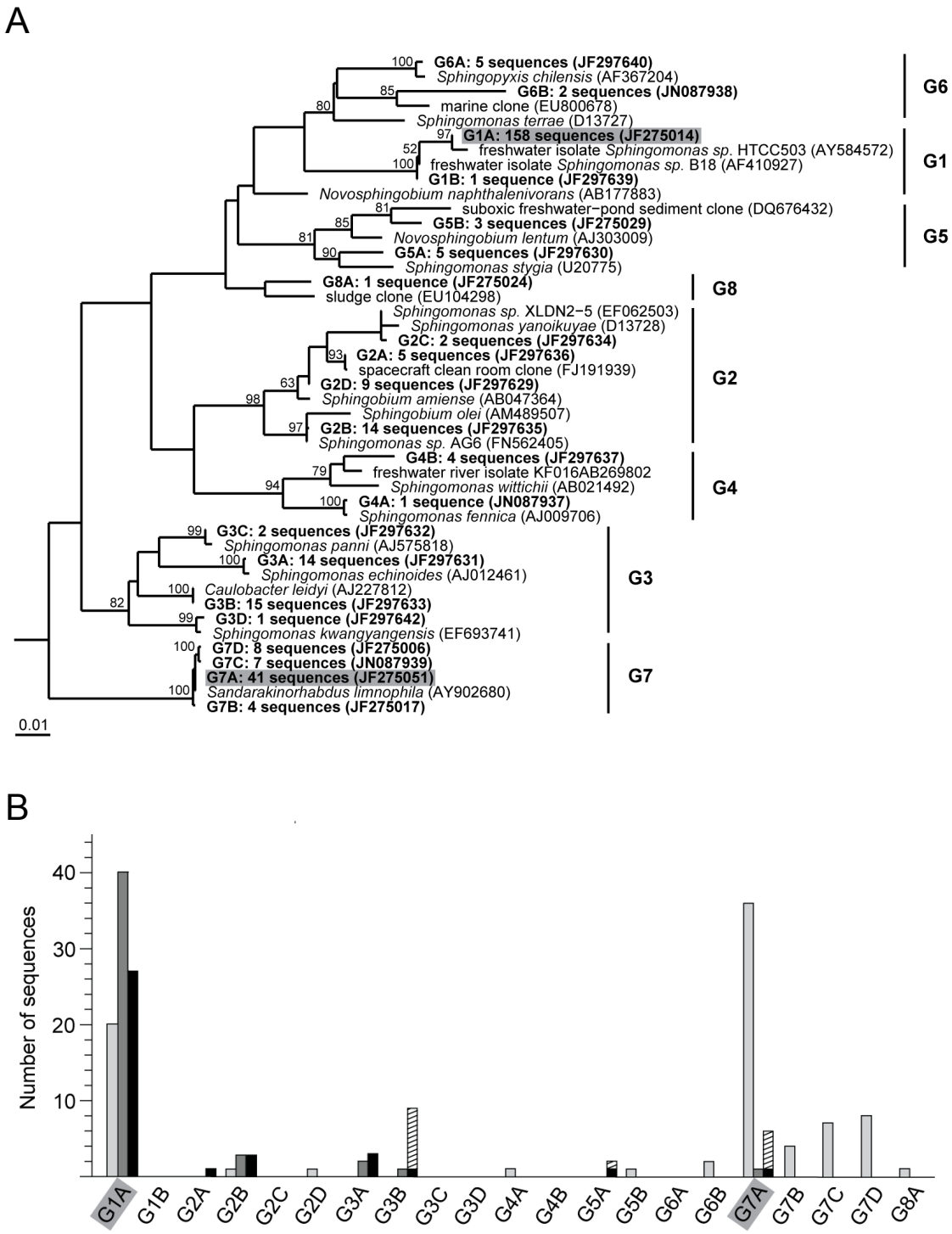

Figure 2 A. Maximum likelihood phylogenetic tree of almost full-length *Sphingomonadaceae* 16S rRNA sequences obtained in the present study (bold). 80 sequences of the environmental clone library, 117 sequences originating from primary liquid enrichment cultures, and 111 sequences of isolated pure cultures of *Sphingomonadaceae* were included in the analysis. The most abundant phylotypes G1A and G7A are indicated by grey boxes. Bar represents 0.01 fixed-point mutations per nucleotide. Values at nodes give bootstrap values in % (out of 1,000 bootstrap resamplings; only values ≥ 50% are given). **B.** Frequency of 16S rRNA sequence types from Walchensee present in the clone library (light grey columns) and enrichments (dark grey columns) (both from December 2007 samples), and among pure isolates from December 2007 (black columns) and August 2008 (hatched columns).

The value for the richness estimator Chao1 calculated for the *Sphingomonadaceae* 16S rRNA gene clone library amounted to 50 phylotypes. Thus, the 11 phylotypes detected in this study account for ~22% of the existing phylotypes. However, because of the skewed frequency distribution that encompassed 2 dominant and many unique sequence types, it appears likely that rare phylotypes account for most of the *Sphingomonadaceae* diversity in Walchensee that was missed by cloning and sequencing.

3.5.2 The two dominant *Sphingomonadaceae* phylotypes persist and are physiologically active throughout different seasons

In a subsequent step, comparative phylogenetic fingerprinting was conducted to elucidate the seasonal shifts in the composition of planktonic *Sphingomonadaceae*. In parallel, cDNA was generated from total RNA extracts and also subjected to phylogenetic fingerprinting in order to identify the physiologically active members of the *Sphingomonadaceae*. Bacterioplankton communities of oligotrophic Walchensee and of neighboring mesotrophic Starnberger See were analyzed during four seasons (Fig. 3).

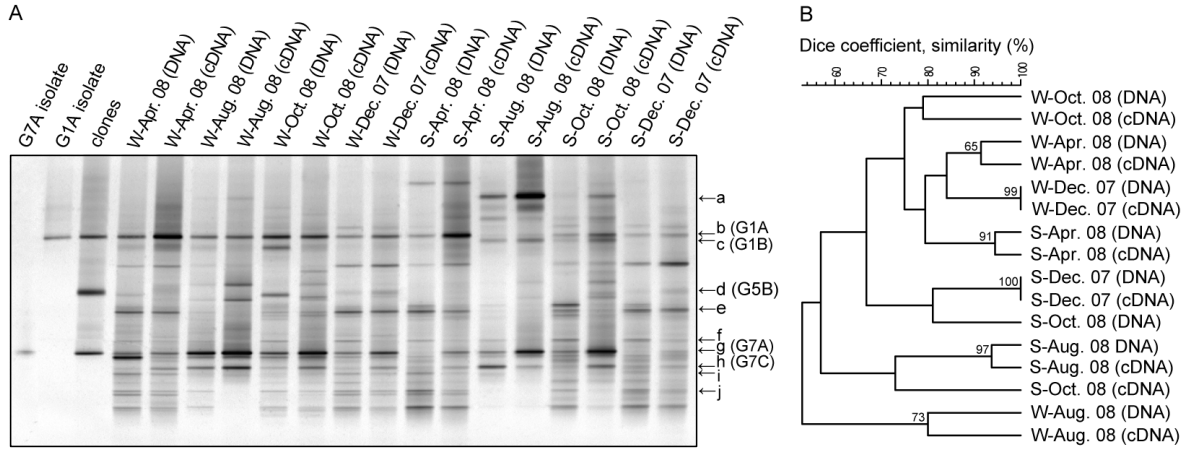

Figure 3 A. Seasonal changes in the composition (as based on analysis of 16S rRNA genes, labeled "DNA") and in the composition of the active fraction (based on analysis of rRNA-cDNA, labeled "cDNA") of *Sphingomonadaceae* in Walchensee and Starnberger See. A negative image of a SybrGold stained denaturing gradient gel is shown. Fragments were amplified using a PCR protocol specific for *Sphingomonadaceae* 16S rRNA genes. For comparison, the fingerprints of an isolated representative of phylotype G1A (isolate 505) and phylotype G7A (isolate 407), as well as fingerprints of cloned sequences of phylotypes G1A, G5B and G7A (compare Fig. 2A) are shown. **B.** Cluster analysis of DGGE fingerprint patterns of seasonal *Sphingomonadaceae* communities using UPGMA. Values at nodes give bootstrap values in % (out of 10,000 bootstrap resamplings; only values ≥ 50% are given). W, Walchensee, S, Starnberger See.

Among the 16S rRNA gene fragments and 16S rRNA-cDNA of *Sphingomonadaceae* separated by denaturing gradient gel electrophoresis, two different fingerprints showed high signal strengths and were found to be present throughout all four seasons in Walchensee (Fig. 3A). The excised bands yielded sequences that were identical to phylotypes G1A and G7A. This corroborates the conclusion that both phylotypes are abundant in Walchensee and also suggests that the corresponding *Sphingomonadaceae* are constantly present. Most notably, the corresponding fragments of G1A and G7A were concomitantly detected in the cDNA, indicating that both phylotypes constitute physiologically active members of the bacterioplankton community throughout the year. Interestingly, both phylotypes were also repeatedly detected and found to be active in the bacterioplankton community of mesotrophic Starnberger See, albeit not during all seasons (Fig. 3A). In Starnberger See, phylotype G1A was barely detectable during summer, whereas G7A was absent in winter. Out of the 10 melting types analyzed by sequencing, five (G1A, G1B, G5B, G7A, G7C) were found to correspond to phylotypes detected in the 16S rRNA gene clone library of Walchensee (Fig. 3A). In addition, five additional melting types of *Sphingomonadaceae* were recovered by the DGGE approach (compare Table S1 in the supplemental material). These melting types either were absent in Walchensee (melting type *a*, Fig. 3A), showed a low overall abundance in Walchensee (*f*, *i*, *k*) or were not present during all seasons (*e*) which provides an explanation for the absence of the corresponding sequences in the clone library generated from this lake. The DGGE clearly separated DNA fragments of phylotypes G7A and G7C that differed by only one base pair (compare Fig. 2A). This provides additional evidence for the sequence difference between both phylotypes (depicted in Fig. 2A) and emphasizes that the DGGE technique is suitable for separation of fingerprints originating from very closely related phylotypes (Muyzer *et al.*, 1993).

Based on a cluster analysis of the DGGE band patterns, DNA and cDNA fingerprints generated for the same water sample were most similar in the majority of cases (Fig. 3B). In fact, DNA and cDNA fingerprint patterns generated for the winter bacterioplankton were virtually identical in Walchensee and Starnberger See, respectively. These results suggest that many of the novel phylotypes of aquatic *Sphingomonadaceae* that were detected in the present study do not represent dormant or dead, but rather ribosome-containing and hence physiologically active constituents of the bacterioplankton communities.

3.5.3 Cultivation of the dominant *Sphingomonadaceae* phylotypes

For the targeted isolation of the dominant *Sphingomonadaceae* phylotypes G1A and G7A, a high throughput cultivation approach in diluted artificial freshwater medium was combined with PCR-based screening of the generated cultures. In order to recover isolates of potentially larger phenotypic diversity, cultures were inoculated with samples from both lakes that were obtained in summer as well as winter.

In total, 1,403 primary liquid cultures were obtained and screened for the presence of *Sphingomonadaceae*. The overall cultivation efficiency determined for bacterioplankton in winter 2007 was (0.40 ± 0.05)% and (0.56 ± 0.07)% for Walchensee and Starnberger See, respectively. In samples obtained from Starnberger See in the following summer 2008 culturability was (0.36 ± 0.05)% and thus remained in the same range, whereas the corresponding values for Walchensee increased to (2.80 ± 0.035)% (Fig. 4). These culturability values are comparable to results of a previous study in which the same medium and a comparable inoculation technique were used (Bruns *et al.*, 2003a).

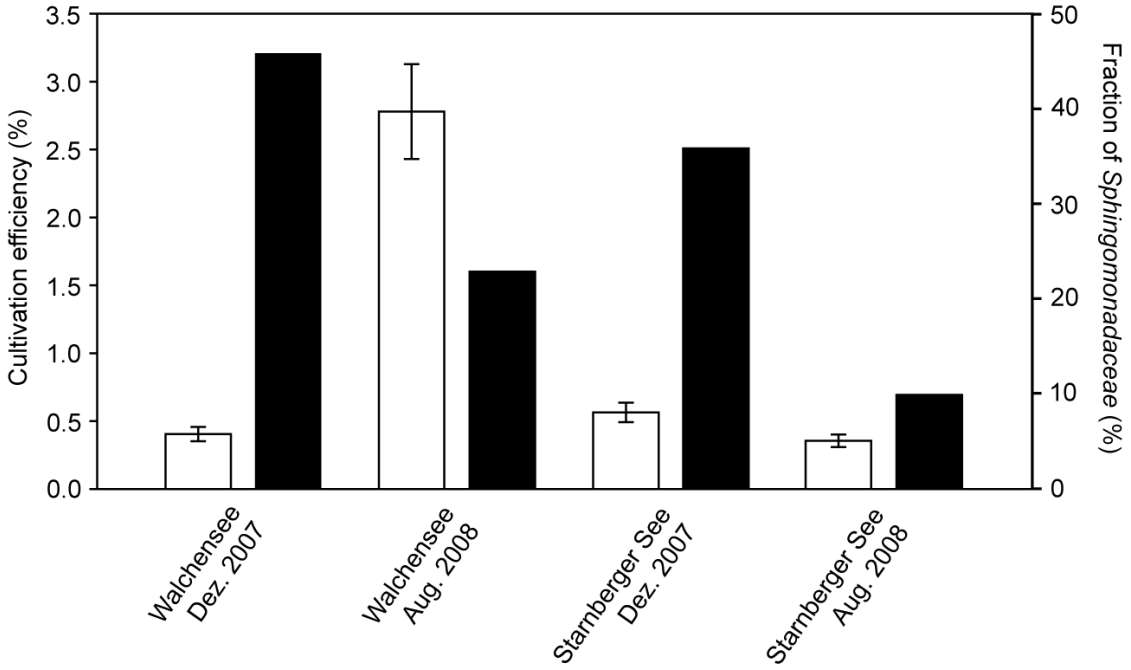

Figure 4. Cultivation success of planktonic bacteria (white columns, left ordinate) and percentage of *Sphingomonadaceae* cultures among the primary enrichments (black columns, right ordinate) derived from bacterioplankton communities in Walchensee and Starnberger See. Cultivation efficiency is given as the percentage of total bacterial cell counts. Error bars indicate 95% confidence intervals.

Employing the specific PCR screen developed for *Sphingomonadaceae*, between 10 and 46% of the positive primary liquid cultures were identified as *Sphingomonadaceae* (Fig. 4). 16S rRNA gene amplicons were generated from the 117 cultures of *Sphingomonadaceae*

obtained from the winter samples, sequenced and subsequent phylogenetically analysed. This revealed the presence of 19 different phylotypes (5 phylotypes present in Walchensee are shown in Fig. 2B; six additional rare phylotypes that neither occurred in the clone library nor among pure cultures are listed in Table S2 in the supplemental material). Most notably, 84 and hence the majority (71%) of enrichments harbored phylotype G1A, whereas only one culture of phylotype G7A was detected. During subsequent isolation attempts, it was observed that bacteria of the G1A phylotype grew only slowly on solid media, and only after agar concentrations had been decreased to 0.8 %. Still, the improved agar media did not permit the isolation of pure cultures from all positive primary enrichments. Our attempts led to 54 pure cultures that were affiliated with the G1A phylotype and an additional single culture of G7A. Although the G1A phylotype was similarly present and active in summer samples (compare Fig. 3A), this phylotype was almost absent in the corresponding primary enrichments and accordingly could not be obtained in pure culture. In contrast, 5 additional pure cultures belonging to the G7A phylotype could be recovered from the summer samples (Fig. 2B). The difference in cultivation success observed for equally active *Sphingomonadaceae* in winter and summer samples may either be the result of a different physiological status of the bacterial cells or of the presence of physiologically different bacterial lineages in the two different seasons. In order to elucidate the presence of different subpopulations, the diversity of internal transcribed spacer regions was analyzed in the natural populations and among the isolated strains.

3.5.4 Identification of ITS1-subpopulations within the G1A phylotype and ecological evidence for niche partitioning

The considerable number of cultures of the same 16S rRNA phylotype that could be recovered in the present study was used to analyse the population substructure of the G1A phylotype. The ITS1 segment of the *rrn* operon was chosen for this analysis since its sequence variability significantly surpasses that of the 16S rRNA genes in most bacteria (Barry *et al.*, 1991; Martiny *et al.*, 2009). An initial comparison of ITS1 sequences of all 54 isolates and of the 20 environmental G1A clones (compare Fig. 2B) revealed the presence of 7 distinct sequence types among the cultured and cloned representatives of G1A (Table S3 in the supplemental material). Among the cultures and clones 5 and 4 different ITS1 types were detected respectively. This diversity already surpasses that detected by intergenic spacer analysis among isolates of the marine *Alphaproteobacterium* `*Candidatus* Pelagibacter ubique´ that demonstrated the existence of three distinct ITS lineages (Rappé *et al.*, 2002). In

fact, our two observations that congruence between the cultured and cloned G1A representatives was limited to 2 sequence types and that many of the sequence types were detected at low frequency is indicative of a larger diversity in the natural G1A population.

In order to cover the ITS1 diversity in Walchensee and Starnberger See more adequately, we used a Illumina sequencing approach and employed primers that amplified the most variable center portion of the ITS1 segment selectively for G1A. DNA and cDNA samples obtained in the four seasons were analyzed separately by multiplexing to follow the seasonal dynamics of individual ITS types and their activity in both lakes.

Due to the rigid quality control used (compare Materials and Methods) the remaining dataset comprised a total of 8,576 ITS1 sequences. This high throughput sequencing uncovered the presence of a significantly larger ITS1 diversity than observed among the cultures and the clone library (Table S3 in the supplemental material). The subsequent analysis of temporal shifts was limited to the 15 ITS1 sequence types that surpassed a value of relative abundance of 5% at least on one of the four sampling dates. This revealed strong seasonal shifts in the abundance of the ITS1-types, which followed an unexpected large number of temporal abundance patterns (Fig. 5). Notably, the G1A population in mesotrophic Starnberger See comprised 8 ITS-types that fluctuated in their relative abundance (Fig. 5B) while the G1A population in Walchensee was strongly dominated by ITS1-type 7 (constituting 64 % of the ITS1 sequences), consistent with the results of our cloning approach (Table S3 in the supplemental material). Furthermore some lake specific ITS types were detected (for Walchensee type L, M, C and Starnberger See type H and 2). The much higher diversity of temporal patterns in Starnberger See may be caused by a larger number of ecological niches available to G1A *Sphingomonadaceae* in this different environment (Table 1) and/or a more dynamic competition between the different ITS1-types of G1A. Niche partitioning has been shown to occur on different levels of diversity in marine *Prochlorococcus*, where 16S rRNA phylotypes that differ by 3% sequence divergence occupy either high or low light intensity niches in the water column of tropical or subtropical oceans (Rocap *et al.*, 2003), and the more closely related (high light-adapted) strains partition themselves according to ambient temperature (Martiny *et al.*, 2009).

Members of G1A were detected in an active state (i.e., in the cDNA sample) only in the December and April samples from Walchensee and in the April sample from Starnberger See (Fig. 5C). As for the DNA sample, ITS1-type 7 dominated the the cDNA from Walchensee; the failure to detect G1A among the active fraction in summer and autumn is consistent with the concomitant decline of this population during these month (Fig. 5A). Similarly, the ITS1

types D and F that dominated in Starnberger See in Spring also constituted the dominant fraction in the cDNA sample at this time (Fig. 5C).

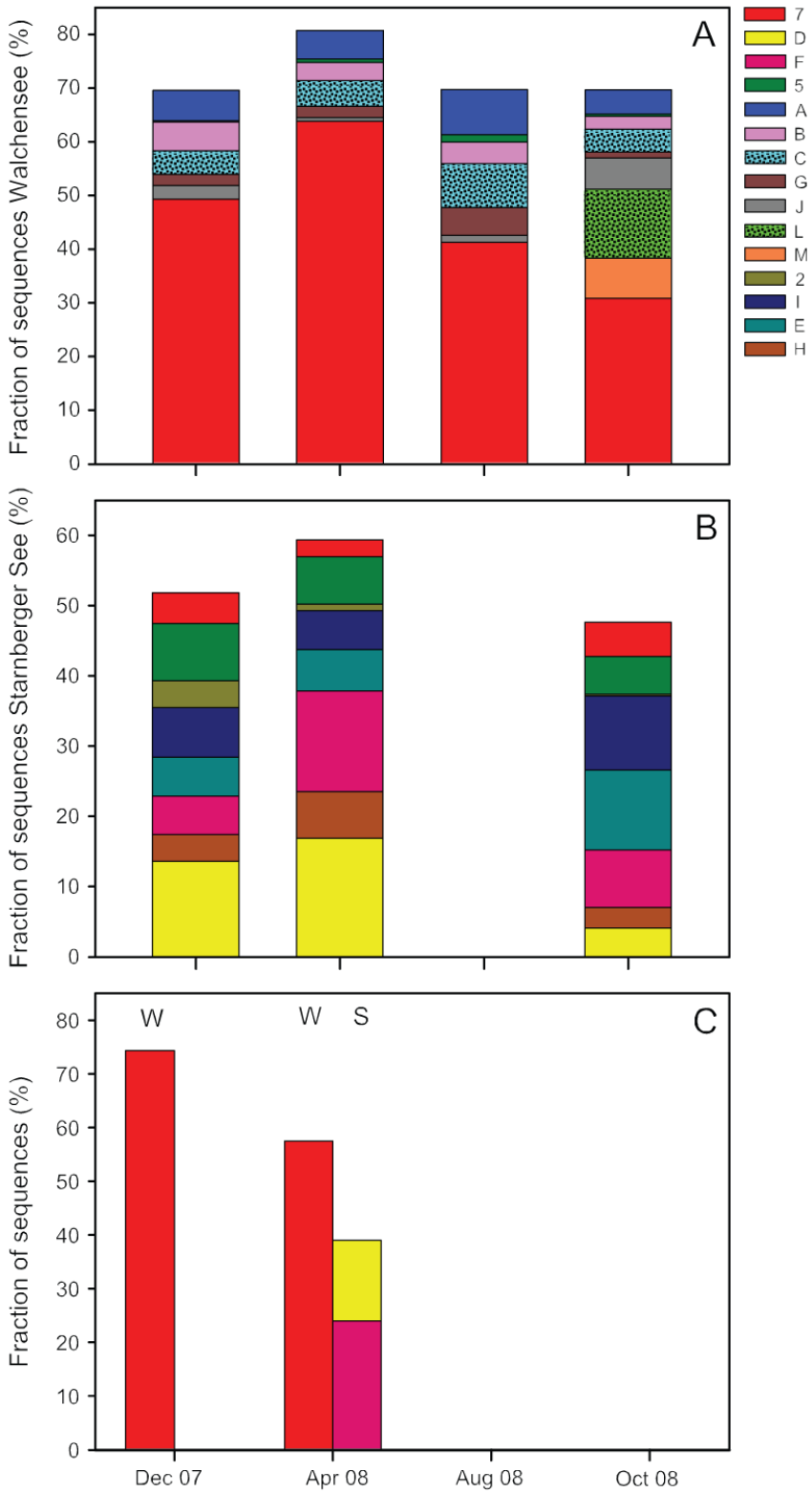

Figure 5. Annual fluctuations of G1A ITS types in
A. Walchensee and **B.** Starnberger See. **C.** Active fraction of G1A ITS1-types detected in cDNA samples. The remaining ITS types were present at a relative abundance <5 % and are not shown.

3.5.5 A high physiological diversity resides within individual ITS1-subpopulations of the G1A phylotype

In some bacteria, ITS sequences have too little resolution to define all ecotypes in a natural population (Melendrez *et al.*, 2011). In a last approach, we therefore analyzed physiological difference within and between the different ITS1-types of G1A cultivated. Sixteen randomly chosen isolates belonging to the ITS1-type 4, and 13 representatives of the four other types (type 2, 3, 5, 6) were selected for testing to obtain comparable sample sizes. The substrate utilization pattern as revealed by the BiOLOG assay clearly showed that the variance of substrate utilization within the 16 isolates with identical ITS sequence (type 4) was even higher than among all other different ITS types combined (Fig. 6). No distinct patterns of substrate utilization could be detected for the different ITS1-types and no differences with respect to the origin of the isolates from the two lakes were observed. 24 different substances of which 80 % could be identified as carbon sources and 20 % as inhibitory substances (antibiotics, low pH) exhibited the highest variance within type 4 (denoted as triangles in Fig. 6; Table S4 in the supplemental material). The four other ITS1-types showed a high variance only for six conditions (4 carbon sources, 2 inhibitors; Table S4 in the supplemental material). The substrate group that exhibited the highest variability in utilization was sugars plus sugar alcohols (42% of the substrates). In addition, very high variance values were detected for resistance against the antibiotics vancomycin and rifamycin, were rifamycin showed the highest variance value of all tested substrates and inhibitors (Suppl. Tab. 5).

Bacterial strains of the same ITS1-type 4 differed considerably with respect to many central metabolic properties. Similar to the situation in *Bacillus simplex* (Sikorski *et al.*, 2008), standard physiological parameters that typically are employed as one of the elements of species delineation therefore do not provide information about possible mechanisms of niche separation that could underlay the differences in abundance patterns of *Sphingomonadaceae* observed in the natural environment. Instead, potential mechanism of niche adaptation in *Sphingomonadaceae* might include among others the degradation of refractory high-molecular-weight organic compounds (Balkwill *et al.*, 2006), resistance to UV radiation (Hörtnagl *et al.*, 2011), a planktonic or sessile life style (Balkwill *et al.*, 2006; Blom & Pernthaler, 2010; Schweitzer *et al.*, 2011), differences in the adaptation to different growth-limiting substrates (Eguchi *et al.*, 1996; Pinhassi & Hagström, 2000; Vancanneyt *et al.*, 2001), aerobic anoxygenic phototrophy (Gich & Overmann, 2006; Kim *et al.*, 2007), or a different susceptibility towards bacteriophage attack (Wolf *et al.*, 2003). Future research

along these lines is needed to elucidate the mechanisms that maintain the large variety of different ITS1 subpopulations of *Sphingomonadaceae* detected in the present study.

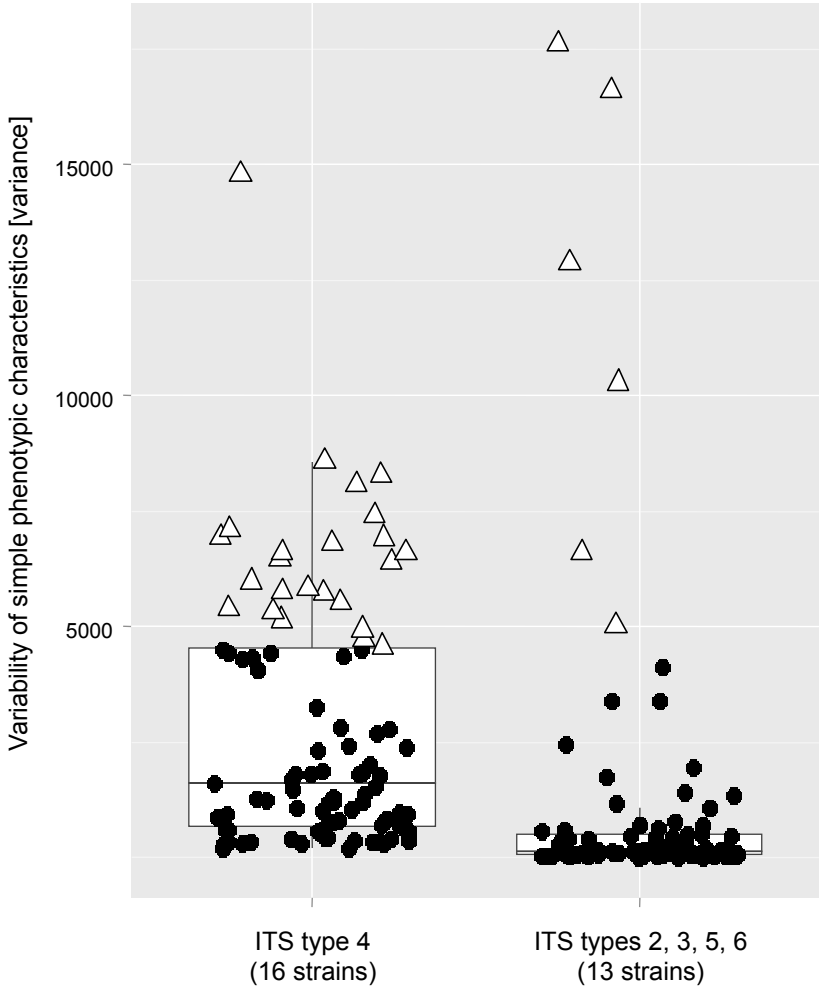

Figure 6. Variances of individual phenotypic traits. The box indicates the first and third quartile, the horizontal line in the box the median value, and the whiskers extend to a maximum of 1.5fold of the box size. The values above the threshold value of the third quartile of ITS type 3 strains are depicted as open triangles for all data. The highly variable substances coded by the open triangles are listed in Suppl. Tab. 5.

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3.7 References

Acinas, S. G., Klepac-Ceraj, V., Hunt, D. E., Pharino, C., Ceraj, I., Distel, D. L. & Polz, M. F. (2004). Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* **430**, 551-554.

Alfreider, A., Pernthaler, J., Amann, R., Sattler, B., Glockner, F., Wille, A. & Psenner, R. (1996). Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. *Appl Environ Microbiol* **62**, 2138-2144.

Altschul, S. F. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Balkwill, D. L., Frederickson, J. K. & Fromine, M. F. (2006). *Sphingomonas* and related genera. *Prokaryotes* **7**, 605-629.

Barry, T., Colleran, G., Glennon, M., Dunican, L. K. & Gannon, F. (1991). The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *Genome Res* **1**, 51-56.

Bartscht, K., Cypionka, H. & Overmann, J. (1999). Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. *FEMS Microbiol Ecol* **28**, 249-259.

Blom, J. F. & Pernthaler, J. (2010). Antibiotic effects of three strains of chrysophytes (*Ochromonas*, *Poterioochromonas*) on freshwater bacterial isolates. *FEMS Microbiol Ecol* **71**, 281-290.

Bruns, A., Cypionka, H. & Overmann, J. (2002). Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central baltic sea. *Appl Environ Microbiol* **68**, 3978-3987.

Bruns, A., Hoffelner, H. & Overmann, J. (2003a). A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. *FEMS Microbiol Ecol* **45**, 161-171.

Bruns, A., Nübel, U., Cypionka, H. & Overmann, J. (2003b). Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. *Appl Environ Microbiol* **69**, 1980-1989.

Cock, P. J. A., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., Friedberg, I., Hamelryck, T., Kauff, F. & other authors (2009). Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25**, 1422-1423.

Cohan, F. M. (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457-487.

Connor, N., Sikorski, J., Rooney, A. P., Kopac, S., Koeppel, A. F., Burger, A., Cole, S. G., Perry, E. B., Krizanc, D. & other authors (2010). Ecology of speciation in *Bacillus*. *Appl Environ Microbiol* **76**, 1349-1358.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. & Relman, D. A. (2005). Diversity of the human intestinal flora. *Science* **308**, 1635-1638.

Eguchi, M., Nishikawa, T., MacDonald, K., Cavicchioli, R., Gottschal, J. & Kjelleberg, S. (1996). Responses to stress and nutrient availability by the marine ultrabacterium *Sphingomonas* sp. strain RB2256. *Appl Environ Microbiol* **62**, 1287-1294.

Eichler, S. Eichler, S., M. G. Weinbauer, K. Dominik, and M. G. Höfle. (2008). Extraction of total RNA and DNA from bacterioplankton, p. 103-120. In G. A. Kowalchuk, de Bruijn, F.J., Head, I.M., Akkermans, A.D., and van Elsas, J.D. (eds) (ed.), Molecular Microbial Ecology Manual, 2nd ed. Springer, Dordrecht, the Netherlands.

Ettema, T. J. G. & Andersson, S. G. E. (2009). The *Alphaproteobacteria*: the Darwin finches of the bacterial world. *Biol Lett* **5**, 429-432.

Fraser, C., Hanage, W. P. & Spratt, B. G. (2007). Recombination and the nature of bacterial speciation. *Science* **315**, 476-480.

Fuhrman, J. A., Comeau, D. E., Hagstrom, A. & Chan, A. M. (1988). Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. *Appl Environ Microbiol* **54**, 1426-1429.

Gich, F. & Overmann, J. (2006). *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a novel bacteriochlorophyll a-containing, obligately aerobic bacterium isolated from freshwater lakes. *Int J Syst Evol Microbiol* **56**, 847-854.

Gich, F., Schubert, K., Bruns, A., Hoffelner, H. & Overmann, J. (2005). Specific detection, isolation and characterization of selected, previously uncultured members of freshwater bacterioplankton. *Appl Environ Microbiol* **71**, 5908-5919.

Giovannoni, S. (2004). Oceans of bacteria. *Nature* **430**, 515-516.

Glöckner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R. (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. *Appl Environ Microbiol* **66**, 5053-5065.

Hörtnagl, P., Pérez, M. T. & Sommaruga, R. (2011). Contrasting effects of ultraviolet radiation on the growth efficiency of freshwater bacteria. *Aquat Ecol* **45**, 125-136.

Jaspers, E. & Overmann, J. (2004). Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. *Appl Environ Microbiol* **70**, 4831-4839.

Jaspers, E., Nauhaus, K., Cypionka, H. & Overmann, J. (2001). Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. *FEMS Microbiol Ecol* **36**, 153-164.

Jezbera, J., Jezberová, J., Brandt, U. & Hahn, M. W. (2011). Ubiquity of *Polynucleobacter necessarius* subspecies asymbioticus results from ecological diversification. *Environ Microbiol* **13**, 922-931.

Kim, M. K., Schubert, K., Im, W.-T., Kim, K.-H., Lee, S.-T. & Overmann, J. (2007). *Sphingomonas kaistensis* sp. nov., a novel alphaproteobacterium containing *pufLM* genes. *Int J Syst Evol Microbiol* **57**, 1527-1534.

Koeppel, A., Perry, E. B., Sikorski, J., Krizanc, D., Warner, A., Ward, D. M., Rooney, A. P., Brambilla, E., Connor, N. & other authors (2008). Identifying the fundamental units of bacterial diversity: a paradigm shift to incorporate ecology into bacterial systematics. *Proc Natl Acad Sci U S A* **105**, 2504-2509.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-175. Edited by E. Stackebrandt & M. Goodfellow. Chichester, UK: Wiley.

Ling, L. L., Keohavong, P., Dias, C. & Thilly, W. G. (1991). Optimization of the polymerase chain reaction with regard to fidelity: modified T7, Taq, and vent DNA polymerases. *PCR Methods Appl* **1**, 63-69.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363-1371.

Majewski, J. & Cohan, F. M. (1999). Adapt globally, act locally: the effect of selective sweeps on bacterial sequence diversity. *Genetics* **152**, 1459-1474.

Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15**, 593 - 600.

Marschall, E., Jogler, M., Henßge, U. & Overmann, J. (2010). Large-scale distribution and activity patterns of an extremely low-light-adapted population of green sulfur bacteria in the Black Sea. *Environ Microbiol* **12**, 1348-1362.

Martiny, A. C., Tai, A. P. K., Veneziano, D., Primeau, F. o. & Chisholm, S. W. (2009). Taxonomic resolution, ecotypes and the biogeography of *Prochlorococcus*. *Environ Microbiol* **11**, 823-832.

Melendrez, M. C., Lange, R. K., Cohan, F. M. & Ward, D. M. (2011). Influence of molecular resolution on sequence-based discovery of ecological diversity among *Synechococcus* populations in an alkaline siliceous hot spring microbial mat. *Appl Environ Microbiol* **77**, 1359-1367.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695-700.

Neef, A. (1997). Ph.D. thesis. Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Technische Universität München, Munich, Germany.

Olsen, G. J., Lane, D. J., Giovannoni, S. J., Pace, N. R. & Stahl, D. A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol* **40**, 337-365.

Overmann, J. & Tuschak, C. (1997). Phylogeny and molecular fingerprinting of green sulfur bacteria. *Arch Microbiol* **167**, 302-309.

Overmann, J., Tuschak, C., Froestl, J. M. & Sass, H. (1998). The ecological niche of the consortium "*Pelochromatium roseum*". *Arch Microbiol* **169**, 120-128.

Page, K. A., Connon, S. A. & Giovannoni, S. J. (2004). Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. *Appl Environ Microbiol* **70**, 6542-6550.

Peplies, J., Glöckner, F. O., Amann, R. & Ludwig, W. (2004). Comparative sequence analysis and oligonucleotide probe design based on 23S rRNA genes of *Alphaproteobacteria* from North Sea bacterioplankton. *System Appl Microbiol* **27**, 573-580.

Piccini, C., Conde, D., Alonso, C., Sommaruga, R. & Pernthaler, J. (2006). Blooms of single bacterial species in a coastal lagoon of the southwestern Atlantic Ocean. *Appl Environ Microbiol* **72**, 6560-6568.

Pinhassi, J. & Hagström, Å. (2000). Seasonal succession in marine bacterioplankton. *Aquat Microb Ecol* **21**, 245-256.

Pinhassi, J. & Berman, T. (2003). Differential growth response of colony-forming α- and γ-*proteobacteria* in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Appl Environ Microbiol* **69**, 199-211.

Rappé, M. S., Connon, S. A., Vergin, K. L. & Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630-633.

Rocap, G., Larimer, F., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N., Arellano, A., Coleman, M., Hauser, L. & other authors (2003). Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* **424**, 1042 - 1047.

Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39-67.

Saiki, R., Gelfand, D., Stoffel, S., Scharf, S., Higuchi, R., Horn, G., Mullis, K. & Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.

Salcher, M. M., Pernthaler, J. & Posch, T. (2011). Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12). *ISME J* **5**, 1242-1252.

Schloss, P. D. & Handelsman, J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**, 1501-1506.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H. & other authors (2009). IIntroducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**, 7537-7541.

Schweitzer, B., Huber, I., Amann, R., Ludwig, W. & Simon, M. (2011). α- and β-Proteobacteria control the consumption and release of amino acids on lake snow aggregates. *Appl Environ Microbiol* **67**, 632-645.

Sikorski, J., Pukall, R. & Stackebrandt, E. (2008). Carbon source utilization patterns of *Bacillus simplex* ecotypes do not reflect their adaptation to ecologically divergent slopes in "Evolution Canyon", Israel. *FEMS Microbiol Ecol* **66**, 38-44.

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today* **8**, 152-155.

Takeuchi, M., Hamana, K. & Hiraishi, A. (2001). Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *Int J Syst Evol Microbiol* **51**, 1405-1417.

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E. & Polz, M. F. (2004). Diversity and dynamics of a North Atlantic coastal *Vibrio* community. *Appl Environ Microbiol* **70**, 4103-4110.

Vancanneyt, M., Schut, F., Snauwaert, C., Goris, J., Swings, J. & Gottschal, J. C. (2001). *Sphingomonas alaskensis* sp. nov., a dominant bacterium from a marine oligotrophic environment. *Int J Syst Evol Microbiol* **51**, 73-79.

Vergin, K. L., Tripp, H. J., Wilhelm, L. J., Denver, D. R., Rappé, M. S. & Giovannoni, S. J. (2007). High intraspecific recombination rate in a native population of *Candidatus* Pelagibacter ubique (SAR11). *Environ Microbiol* **9**, 2430-2440.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. (2007). Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261-5267.

Wickham, H. (2009). *ggplot2:* elegant graphics for data analysis. New York: Springer.

Wolf, A., Wiese, J., Jost, G. & Witzel, K.-P. (2003). Wide geographic distribution of bacteriophages that lyse the same indigenous freshwater isolate (*Sphingomonas* sp. strain B18). *Appl Environ Microbiol* **69**, 2395-2398.

Zwart, G., Crump, B. C., Kamst-Van Agterveld, M. P., Hagen, F. & Han, S. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**, 141-155.

3.8 Supplementary Figures

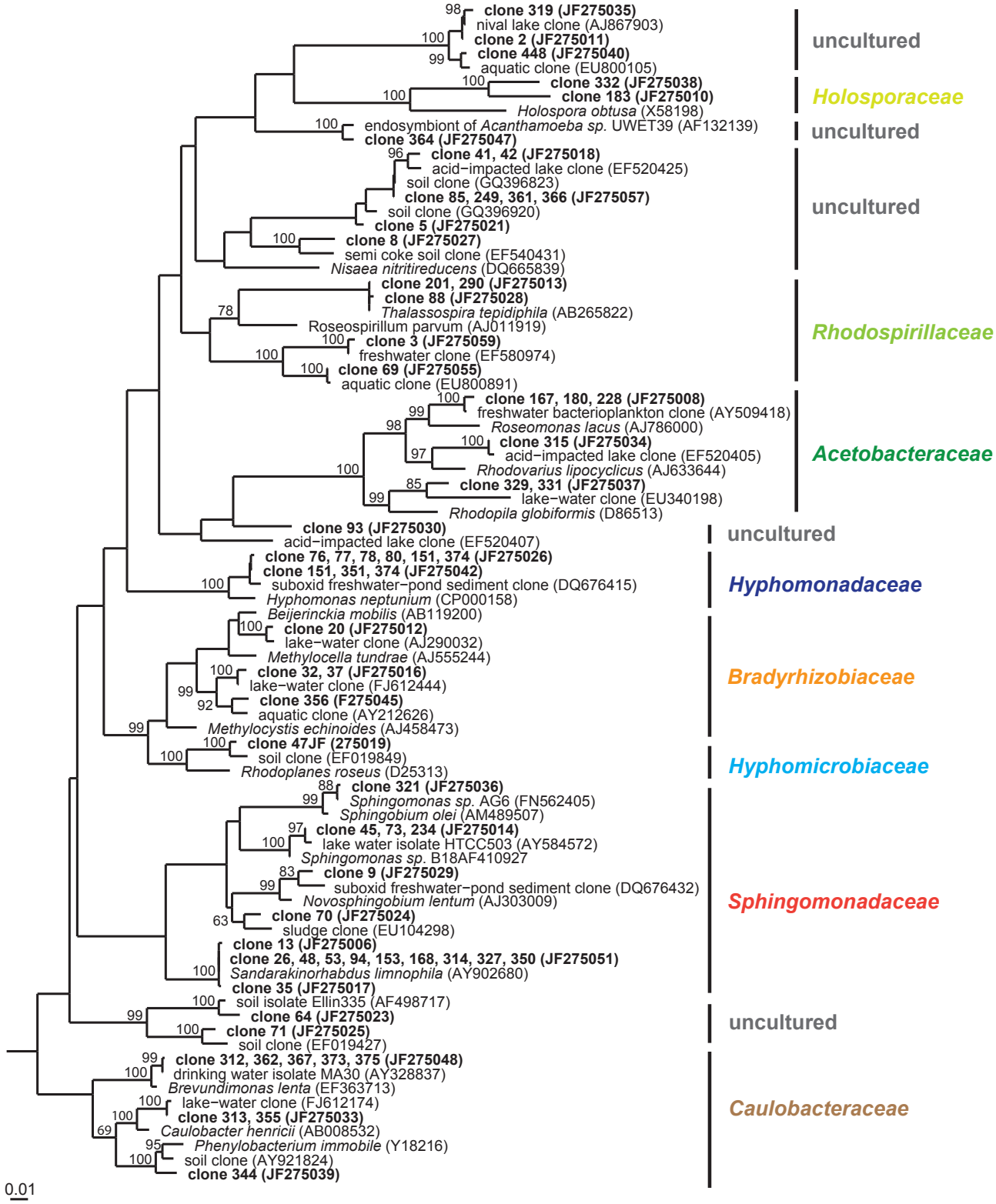

Figure S1. Maximum likelihood phylogenetic tree of 66 *Alphaproteobacteria* 16S rRNA gene sequences cloned from four bacterioplankton communities of lake Walchensee (depicted in bold). The closest relatives of all sequences are included in the analysis. Bar represents 0.01 fixed-point mutations per nucleotide. Values at nodes give bootstrap values in % (out of 1,000 bootstrap resamplings; only values ≥ 50% are given).

3.9 Supplementary Tables

Supplementary Table S1. Classification of the *Sphingomonadaceae* DGGE bands. For each melting type the closest related GenBank match is provided together with the corresponding type strain and the closest 16S rRNA gene sequence originating either from the clone library or from isolates obtained in the present study.

melting type	closest GenBank match	identity	closest type strain	identity	closest phylotype (this study)	identity
a	groundwater isolate K39 (AJ009707)	99.6 %	*Sphingomonas stygia* 12445^{T} (U20775)	98.65 %	G5B (JF275029)	98.7 %
b	lake water isolate HTCC503 (AY584572)	99.5 %	*Novosphingobium naphthalenivorans* 18518^{T} (AB177883)	93.0 %	G1A (JF270514)	**100 %**
c	subsurface water clone	98.6 %	*Novosphingobium naphthalenivorans* 18518^{T} (AB177883)	95.1 %	G1B (JF297639)	**100 %**
d	glacier clone (DQ228401)	99.3 %	*Novosphingobium lentum* 13663^{T} (AJ303009)	98.4 %	G5B (JF275029)	**100 %**
e	*Sphingomonas echinoides* 1805^{T} (AJ012461)	96.7 %	*Sphingomonas echinoides* 1805^{T} (AJ012461)	96.7 %	G3A (JF297631)	96.7 %
f	wastewater sludge digester clone (CU918739)	99.1 %	*Novosphingobium resinovorum* 7478^{T} (EF029110)	95.3 %	G8A (JF275024)	96,8 %
g	*Sandarakinorhabdus limnophila* 17366^{T} (AY902680)	**100 %**	*Sandarakinorhabdus limnophila* 17366^{T} (AY902680)	100 %	G7A	**100 %**
h	*Sandarakinorhabdus limnophila* 17366^{T} (AY902680)	99.77 %	*Sandarakinorhabdus limnophila* 17366^{T} (AY902680)	99.77 %	G7C	**100 %**
i	soil isolate PI_GH4.1.H5 (AY162055)	99.3 %	*Sphingobium olei* 18999^{T} (AM489507)	98.1 %	G2B (JF297635)	98.1 %
j	*Sphingobium amiense* 16289^{T} (AB047364)	99.3 %	*Sphingobium amiense* 16289^{T} (AB047364)	99.3 %	G2D (JF297629)	99.8 %

Supplementary Table S2. 16S phylotypes detected exclusively within primary liquid cultures. None of them was detected in the alphaproteobacterial 16S rRNA gene library nor obtained as pure isolate.

phylotype	closest GenBank match	identity	closest type strain	identity
G1	freshwater bacterioplankton isolate HTCC503 (AY584571)	100 %	*Sphingopyxis flavimaris* 16223^{T} (AY554010)	93.4 %
G1	wastewater sludge digester clone (CU 926003)	99.1 %	*Sphingopyxis alaskensis* 13593^{T} (NC008048)	95.1 %
G2	stalactite carbon cave strain KC-IT-H8 (FJ711206)	99.1 %	*Sphingomonas insulae* 21792 ^{T} (EF363714)	96.1 %
G2	hypertrophic lake clone (DQ520195)	99.6 %	*Sphingomonas fennica* 13665^{T} (AJ009706)	91.7 %
G3	*Caulobacter leidyi* 4733^{T} (AJ227812)	99.3 %	*Caulobacter leidyi* 4733^{T} (AJ227812)	97.3 %
G5	*Novosphingobium lentum* 13663^{T} (AJ303009)	98.5 %	*Novosphingobium lentum* 13663^{T} (AJ303009)	98.5 %

Supplementary Table S3. G1A ITS types detected in this study and the variable positions according to the full length sequence.[a]

Type	Illumina reads	Number of Clones	Isolates	ITS positions[a] 63	67	84	98	100	134	136	137	141	143	145	543	557	558	576	577	578
	Prevailing nucleotide			T	T	C	C		C	A	T	G	A	T	G	T	C	G	G	C
7	3491	10	0																	
D	732	0	0		C		T	T	G	G	C	T						A	A	T
F	607	0	0		C		T	T		G	C	T	G							
5	269	0	8		C					G	C	T	G					A	A	T
A	309	0	0	C	C		T	T		G	C	T	G				T	A	A	
B	314	0	0															A	A	T
C	277	0	0														T	A	A	
G	233	0	0	C	C		T	T		G	C	T	G							
J	267	0	0		C		T	T	G	G	C	T								
L	143	0	0		C		T	T	G	G	C	T						A	A	T
M	84	0	0													C		A	A	T
2	53	0	1		C					G	C	T	G		T	C			A	
I	297	0	0	C	C		T	T		G	C	T	G					A	A	T
E	294	0	0	C	C		T	T		G	C	T	G					T	A	T
H	230	0	0		C					G	C	T	G					A	A	T
1	0	2	0		C	T				G	C	T	G		T			A	A	T
3	2	0	5	C	C		T	T		G	C	T	G					A	A	T
4	0	2	36		C		T		G	G	C	T						A	A	T
6	0	6	4	C	C		T			G	C	T	G				T	A	A	

[a] empty fields correspond to prevailing nucleotide given in the first row

Supplementary Table S4. Individual phenotypic characteristics of high variability

ITS type	Variance value[1]	Substance[2]	Type of substance
4	4563	Citric Acid	carbon source
4	4708	Glycyl-L-Proline	carbon source
4	4934	Formic Acid	carbon source
4	5133	pH 5 *	inhibitory substance
4	5310	D-Mannitol	carbon source
4	5384	Nalidixic Acid	inhibitory substance
4	5535	D-Sorbitol	carbon source
4	5711	D-Mannose	carbon source
4	5755	Acetic Acid	carbon source
4	5829	a-D-Glucose	carbon source
4	5972	D-Maltose	carbon source
4	6381	L-Alanine	carbon source
4	6462	D-Trehalose	carbon source
4	6596	D-Fructose	carbon source
4	6605	Tetrazolium Blue *	inhibitory substance
4	6787	Aztreonam	inhibitory substance
4	6910	D-Cellobiose	carbon source
4	6935	g-Amino-Butryric Acid	carbon source
4	7093	D-Arabitol	carbon source
4	7409	Inosine	carbon source
4	8072	Vancomycin *	inhibitory substance
4	8278	L-Galactonic Acid Lactone	carbon source
4	8579	N-Acetyl-D-Glucosamine	carbon source
4	14773	Rifamycin SV *	inhibitory substance
2, 3, 5, 6	5015	D-Trehalose	carbon source
2, 3, 5, 6	6591	Vancomycin *	inhibitory substance
2, 3, 5, 6	10271	Rifamycin SV *	inhibitory substance
2, 3, 5, 6	12867	L-Arginine	carbon source
2, 3, 5, 6	16578	pH 5 *	inhibitory substance
2, 3, 5, 6	17602	Tetrazolium Blue *	inhibitory substance

[1] Variance values are shown in Figure 6 as open triangles

[2] Substances marked with an asterisk (*) are highly variable in both ITS type 4 and in the pooled ITS types 2, 3, 5, 6.

Chapter 4

Sphingorhabdus planktonica gen. nov., sp. nov., an abundant member of freshwater bacterioplankton communities

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4.1 Contribution of the author

Strain G1A_585^T was isolated by Mareike Jogler and maintained together with Hong Chen. For polar lipid and fatty acid profiles the biomass was produced and interpreted by Mareike Jogler, whereas the determination was conducted by Gabriele Pötter.

Mareike Jogler and Julia Simon performed biomass production for the determination of polyamine and G+C content. Mareike Jogler accomplished phase contrast micrographs, determination of cell size and phylogenetic analysis. Mareike Jogler conducted growth tests with assistance of Anja Frühling. Mareike Jogler and Jörg Overmann wrote the article with support of Hans-Jürgen Busse. All figures and Tables were conducted by Mareike Jogler and Jörg Overmann.

4.2 Abstract

A novel type of an aerobic, non-sporulating bacterium, strain G1A_585^T, was isolated from an oligotrophic freshwater lake in Bavaria (Germany). The rod-shaped cells stained gram-negative and were non-motile. Based on 16S rRNA gene similarity strain G1A_585^T is a member of the family *Sphingomonadaceae* and shared less than 95.2 % similarity with all type strains of the most closely related genus *Sphingopyxis*. Polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidyl-monomethylethanolamine, phosphatidyldimethyl-thanolamine, phosphatidylcholine, sphingoglycolipids, three glycolipids and one unknown lipid. Ubiquinone-10 represents the dominant quinone (93.1 %) and is accompanied by ubiquinone-9 (6.5 %). The major cellular fatty acids are 18:1*ω7c* and/or 18:1*ω6c* (summed feature 8) (38.2%); 16:1*ω7c*, 16:1*ω6c* and/or 15:0 iso 2-OH (summed feature 3) (33.6%) and 14:0 2-OH (17.8%). As the major polyamine spermidine and traces of 1,3-diaminopropane, putrescine and spermine were detected. Strain G1A_585^T has a DNA G+C content of 55.7 mol% and tested positive for oxidase and catalase. Based on the phylogenetic distance, the composition of fatty acids and the low G+C content a novel genus and species, *Sphingorhabdus planktonica* gen. nov. and sp. nov. is proposed with strain G1A_585^T (DSM 25081^T) as the type strain.

4.3 Introduction, Methods, Results and Discussion

Members of the family *Sphingomonadaceae* represent typical freshwater bacteria (Gich *et al.*, 2005; Glöckner *et al.*, 2000; Zwart *et al.*, 2002), but also occur widespread in the marine environment, in pristine and contaminated soils, the rhizosphere and in clinical specimen (Balkwill *et al.*, 2006; Kim *et al.*, 2007; Takeuchi *et al.*, 2001). The genus *Sphingomonas* was established in 1990 (Yabuuchi *et al.*, 1990) and later divided into the four genera *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi *et al.*, 2001). In addition to well known genera *Blastomonas* and *Zymomonas*, several novel genera were recently established comprising *Sandaracinobacter*, *Sphingosinicella*, *Sandarakinorhabdus*, and most recently *Stakelama* (Chen *et al.*, 2010) and *Sphingomicrobacterium* (Kämpfer *et al.*, 2011). *Sphingomonadaceae* are characterized by harboring glycosphingolipids (Yabuuchi *et al.*, 1990) and are physiologically highly diverse. Several members are capable of aerobic anoxygenic photosynthesis (Kim *et al.*, 2007). Many sphingomonads degrade polycyclic aromatic compounds and xenobiotics (Basta *et al.,* 2005), including toxic dioxin pollutants, which make them useful for industrial wastewater treatment (Wittich *et al.*, 1992). Whereas most *Sphingomonadaceae* are typically aerobic, *Zymomonas mobilis* subspecies are facultative anaerobes that produce ethanol via fermentation (Kalnenieks, 2006).

A survey of bacterioplankton in an oligotrophic alpine freshwater lake (Walchensee, near Kochel in Bavaria, Germany; 802 m above sea level) revealed that *Sphingomonadaceae* constituted the major fraction of *Alphaproteobacteria* (27% of all 16S rRNA gene clones). Among the *Sphingomonadaceae*, one previously unknown 16S rRNA gene sequence type accounted for 25% of the sequences. This phylotype was identified to be physiological active were it was present throughout the year, not only in the oligotrophic Walchensee but also in the nearby mesotrophic Starnberger See (Jogler *et al.*, 2011). Several isolates of this sequence type were isolated from both lakes and a representative, strain G1A_585^T selected for subsequent characterization.

Water samples were obtained from Walchensee on December 20, 2007, from a boat at a distance of 30 m from the western shore (47°35′N, 11°20′E). Strain G1A_585^T was enriched in microtiter plates employing diluted growth medium and a high throughput multidrop technique (Bruns *et al.*, 2003). The medium consists of basic synthetic freshwater medium buffered with 10 mM HEPES (Bartscht *et al.*, 1999) supplemented with 20 canonical amino acids, glucose, pyruvate, citrate, 2-oxoglutarate, succinate (200 μM each), Tween 80 (0.001% v/v) and a fatty acid mixture containing formate, acetate and propionate (200 μM each)

(Jaspers *et al.*, 2001). Trace element solution SL 10 (final concentration, 1 ml·l^{-1}), 10-vitamin solution (final concentration, 10 ml·l^{-1}) and the inducers cAMP, *N*-butyryl homoserine lactone and *N*-oxohexanoyl-DL-homoserine lactone were added at final concentrations of 10 µM each (Bruns *et al.*, 2002).

Strain G1A_585^T was purified from the enrichment cultures by streaking on agar plates containing artificial freshwater, 1:10 diluted HD medium (HD medium consisting of 0.05 % casein peptone, 0.01 % glucose, 0.025 % yeast extract; w/v) and buffered with 10 mM HEPES, pH 7. The agar was washed three times and applied at 1% (w/v) concentration. Growth was also tested on plates containing 1:10 diluted HD medium but without artificial freshwater, as well as on standard R2A, Nutrient and CASO agar (Oxoid), but no growth was observed. Only poor growth of strain G1A_585^T was detected in liquid 1:10 HD medium without artificial freshwater.

Colonies on agar plates appeared yellowish-orange and transparent. Carotenoids were extracted from 10 ml of liquid culture in the early stationary phase. The culture was centrifuged and the cell pellet mixed with 1 ml pure acetone. Absorption spectra were monitored with a Lambda 25 spectrometer (Perkin Elmer). Within the spectrum a shoulder at 427 nm and two peaks at 453 nm and 481 nm were detected, indicating the presence of carotenoids in strain G1A_585^T. Typical carotenoids of *Sphingomonadaceae* are zeaxantin and/or nostoxanthin. A shoulder positioned between 420 nm and 430 nm is typical for other *Sphingomonadaceae* species and due to unidentified pigments (Asker *et al.*, 2007; Busse *et al*., 2003; Denner *et al*., 2001; Gich & Overmann, 2006; Jenkins *et al.,* 1979; Stolz *et al*., 2000).

The 16S rRNA gene was PCR amplified using the primers 8f and 1492r (Lane, 1991). Phylogenetic analysis was conducted with the ARB software package (Ludwig *et al.*, 2004). Sequences were automatically aligned with the integrated Fast Aligner tool of the ARB package, and the alignment was corrected manually according to secondary structure information. Phylogenetic trees were generated with the ARB software package based on the Maximum Likelihood algorithm and bootstrap values were calculated with 1,000 bootstrap resamplings. The 16S rRNA gene sequence of strain G1A_585^T obtained in the present study was deposited in the GenBank database under accession number JN381068. Within the maximum likelihood phylogenetic tree, strain G1A_585^T constitutes a separated branch, yet shares a root with a cluster of the three marine species *Sphingopyxis flavimaris* (DSM 16223^T), *Sphingopyxis marina* (DSM 22363^T) and *Sphingopyxis litoris* (DSM 22379^T) (Fig

1). The common root with the latter species has a moderate bootstrap support of 64%. All type strains of *Sphingopyxis* showed less than 95.2% 16S rRNA gene similarity to strain G1A_585^T.

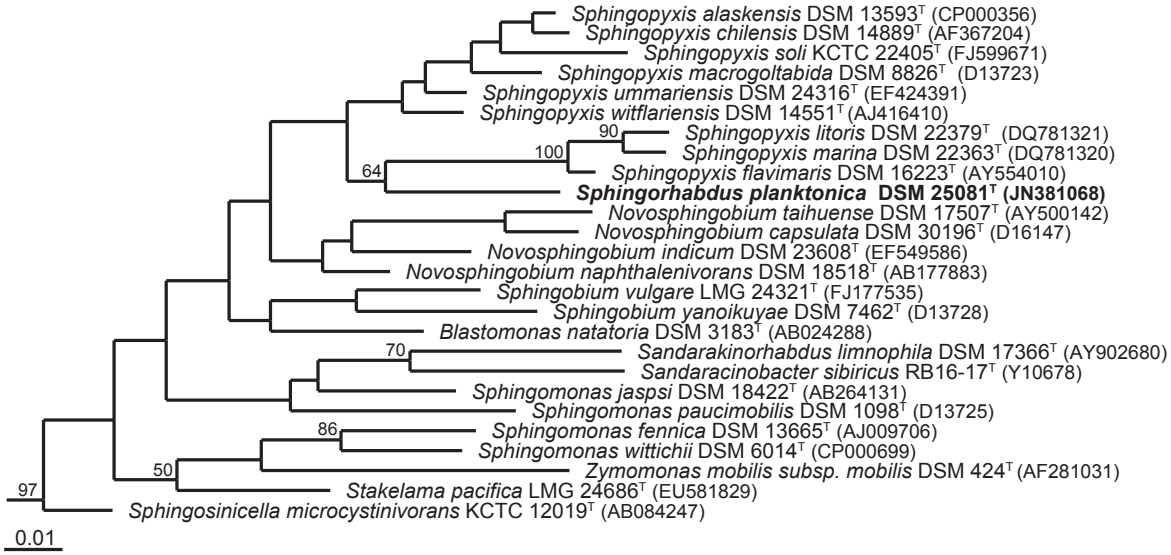

Figure 1. Maximum likelihood phylogenetic tree of *Sphingomonadaceae.* Bar represents 0.01 fixed point mutations per nucleotide. Values at nodes give bootstrap values in % (out of 1,000 bootstrap resamplings; only values ≥ 50% are given).

The rod-shaped cells were on average (1.17 ± 0.20) µm long and (0.47 ± 0.07) µm wide (Fig. S1, supplementary material) as determined with an Axio Imager M2 microscope (Zeiss) equipped with an Axiocam MRm camera and the AxioVision 4.8.2. software (Zeiss). In contrast to members of all validated *Sphingopyxis* species strain G1A_585^T is immotile. Transmission and scanning electron microscopy revealed no flagella (Fig. S1, supplementary material).

Cells stained Gram-negative and were positive for cytochrome *c* oxidase and catalase as tested using standard methods (Gerhardt *et al.*, 1994). For determination of the optimal pH for growth, artificial freshwater medium with 1:10 diluted HD was prepared at different pH using appropriate buffers. MES was employed for pH values of 5, 6 and 6.5; HEPES for 7, 7.5 and 8, HEPPS for 8.5 and CHES for values of pH 9 and 10. Strain G1A_585^T is able to grow between pH 6.5 and pH 9, with optimal growth detected between pH 7 and 8. The temperature optimum of growth was at 18°C to 24°C, whereas no growth was detected below 11°C and above 27°C. The G+C content of genomic DNA of strain G1A_585^T was determined as described previously (Cashion *et al.*, 1977; Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984) and was 55.7 mol%. By comparison, species within the genus *Sphingopyxis*

have higher DNA G+C contents between 62.3-69.2 mol%, with the exception of *Sphingopyxis flavimaris* DSM 16223^T that has a content of 58.0 mol% (Tab. 1). The DNA G+C content from *S. litoris* and *S. marina* are unknown, thus further analysis has to be done to consider that this three *Sphingopyxis* species might be included into the genus *Sphingorhabdus*.

Table 1. Differential phenotypic characteristics of isolate G1A_585^T, related Sphingopyxis species and various genus typestrains.

1, G1A_585^T (this study); 2, *Sphingopyxis witflariensis* DSM 14551^T (Zhang et al., 2010; Srinivasan et al., 2010); 3, *Sphingopyxis flavimaris* DSM 16223^T (Yoon & Oh, 2005); 4, *Sphingopyxis macrogoltabida* DSM 8826^T (Takeuchi et al., 1993; Lee et al, 2008; Srinivasan et al., 2010); 5, *Novosphingobium capsulatum* DSM 30196^T; 6, *Sphingobium yanoikuyae* DSM 7462^T; 7, *Sphingomonas paucimobilis* DSM 1098^T (Yabuuchi et al, 1990; Takeuchi et al., 2001). All strains were positive for katalase and utilization of glucose. All strains were negative for indole production, glucose fermentation, urease, D-mannitol and phenylacidic acid.

	1	**2**	**3**	**4**	**5**	**6**	**7**
color	yellowish-orange transparent[1]	yellow transparent[1]	yellow[2]	yellowish white[1]	yellow[1]	white[1]	yellow[1]
motility	-	+	+	+	-	+	+
oxidase	+	+	+	+	+	+	-
DNA G+C content (mol%)	55.7	ND	58	63.9	63.1	61.7	63.7
reduction of nitrates	-	-	-	-	+	-	+
arginine dihydrolase	-	-	ND	-	-	-	-
β-glucosidase	+	-	ND	+	+	+	+
protease	-	-	-	-	-	-	+
β-galactosidase	+	-	ND	+	+	+	+
utilization of:							
L-arabinose	-	-	-	+	+	+	+
D-mannose	-	-	-	-	+	-	+
N-acetyl-glucosamine	-	-	ND	-	+	+	+
D-maltose	-	+	-	+	+	+	+
potassium gluconate	-	-	ND	-	+	+	-
capric acid	-	+	ND	+	ND	ND	ND
adipic acid	-	-	ND	-	-	-	-
malic acid	-	-	-	-	+	+	+
trisodium citrate	-	-	-	-	-	+	+

[1]analyzed in this study on plates containing 1% washed agar with artificial freshwater and 1:10 HD medium.
[2]according Yoon & Oh, 2005.

Quinones and polar lipids were extracted from 0.1 g of lyophilized biomass (Tindall, 1990a, b). Strain G1A_585^T contains ubiquinone-10 (93.1 %) in addition to minor amounts of ubiquinone-9 (6.5 %). Polar lipids comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine, sphingoglycolipids, three glycolipids and one unknown lipid (Fig. S2, supplementary material). Polyamines were extracted from cells in the stationary growth phase according to the procedure described previously (Busse & Auling, 1988). Analysis was carried out using the HPLC equipment described by Stolz *et al*. (2007). Spermidine was the major polyamine [21.1 µmol (g dry biomass)$^{-1}$]. In addition, traces [<0.6 µmol (g dry biomass)$^{-1}$] of 1,3-diaminopropane, putrescine and spermine were detected. For the fatty acid analysis (Stead *et al.*, 1992) strain G1A_585^T and type species of seven genera (*Sphingopyxis macrogoltabida* DSM 8826^T; *Sphingosinicella microcystinivorans* DSM 19791^T; *Sphingomonas paucimobilis*^{T} DSM 1098; *Sphingobium yanoikuyae* DSM 7462^T; *Novosphingobium capsulatum* DSM 30196^T; *Sandarakinorhabdus limnophila*, DSM 17366^T; *Blastomonas natatoria* DSM 3183^T) and the closest relative *Sphingopyxis witflariensis* (DSM 14551^T) were grown under identical conditions (artificial freshwater with 1:10 HD, 20°C) for better comparability since the fatty acid profile has been shown to vary for cells grown under different conditions (Sikorski *et al.*, 2008; Takeuchi *et al.*, 2001). In addition, the fatty acid composition of strain G1A_585^T was compared to published profiles of all recognized *Sphingopyxis* species based on the LPSN database (http://www.bacterio.cict.fr/), with the exception of *Sphingopyxis marina* DSM 22363^T and *Sphingopyxis litoris* DSM 22379^T for which no detailed fatty acid profiles were published.

The dominant cellular fatty acids were 18:1*ω7c* and/or 18:1*ω6c* (summed feature 8) reaching 38.19 %; 16:1*ω7c*, 16:1*ω6c* and/or 15:0 iso 2-OH (summed feature 3) with 33.55 %, and 14:0 2-OH with 17.75%. In addition 16:0 (8.04%), 16:1 2-OH (1.72%), 14:0 (0.67%), 17:0 (1.46%), 17:1ω6c (1.12%) 16:1ω9c (0.91%), 16:0 2-OH (0.71%) and 15:0 (0.18%) were detected. Interestingly, strain G1A_585^T showed an exceptional high amount of 14:0 2-OH (17.75%) compared to all tested genus type strains (Tab. 2) and all *Sphingopyxis* species (Tab. S1, supplementary material) that contain amounts between 1.3% and 11.05%. Furthermore 16:1ω9c was not detected in all *Sphingopyxis* species and tested genus type strains. All *Sphingopyxis* species exhibit 18:1*ω7c* 11-methyl, that is normally produced from

18:1*ω7c* in the stationary phase. However this fatty acid was not detected in strain G1A_585^T, despite using a stationary culture for this analysis.

Table 2. Fatty acid composition of strain G1A_585, the closest relative *Sphingopyxis witflariensis* 14551^T and various genus type strains after growth in artificial freshwater with 1:10 diluted HD medium.
1, strain G1A_585; 2, *Sphingopyxis witflariensis* DSM 14551^T; 3, Sphingopyxis *macrogoltabida* DSM 8826^T; 4, *Sphingomonas paucimobilis* DSM 1098^T; 5, *Sphingobium yanoikuyae* DSM 7462^T; 6 *Novosphingobium capsulatum* DSM 30196^T; 7, *Blastomonas natatoria* DSM 3183^T; 8, *Sphingosinicella microcystinivorans* DSM 19791^T; 9, *Sandarakinorhabdus limnophila* DSM 17366^T.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
non polar									
14:0	0.67	0.11	0.14	0.17	-	0.11	0.23	0.32	7.6
15:0	0.18	2.93	0.1	-	-	0.22	0.11	-	2.36
16:0	8.04	2.1	6	8.07	5.61	9.94	11.3	2.95	11.13
17:0	1.46	0.82	-	-	-	0.29	0.1	-	-
17:0 cyclo	-	-	-	1.22	-	-	-	-	-
18:0	-	0.1	0.2	0.54	-	0.29	0.29	-	-
19:0 cyclo*ω8c*	-	-	-	0.74	0.89	2.28	-	0.65	-
15:1*ω6c*	-	0.3	-	-	-	-	-	-	-
16:1*ω5c*	-	1.25	1.91	0.63	3.08	1.07	1.79	2.34	1.22
16:1*ω9c*	0.91	-	-	-	-	-	-	-	-
17:1*ω6c*	1.12	22.13	0.79	-	0.96	1.9	2.37	0.49	7.16
17.1*ω8c*	-	4.23	-	-	-	0.24	0.23	-	-
17:1 iso*ω9c*	-	0.05	-	-	-	-	-	-	-
18:1*ω5c*	-	0.28	0.49	1.69	0.79	0.91	0.81	0.45	-
18:1*ω7c* 11-methyl	-	6.16	5.03	-	2.07	-	-	1.6	-
Hydroxy fatty acids									
13:0 2-OH	-	0.09	-	-	-	-	-	-	-
14:0 2-OH	17.75	3.05	1.9	3.34	5.27	11.05	2.63	10.1	4.82
15:0 2-OH	-	8.38	-	-	-	0.37	0.14	0.12	-
16:0 2-OH	0.71	2.35	1.19	-	0.7	-	0.46	0.65	0.77
16:1 2-OH	1.72	0.08	-	-	-	-	-	0.15	-
16:0 iso 3-OH	-	-	0.27	-	-	-	0.79	0.6	-
18:1 2-OH	-	0.05	0.11	-	-	-	-	-	-
Summed features·									
3	30.55	23.3	34.83	3.76	28.43	5.97	21.1	34.39	36.37
7	-	0.19	-	-	-	-	-	-	-
8	38.19	24.97	47.15	79.84	52.24	68.58	57.76	45.09	30.92

Physiological and biochemical characteristics were determined using API 20E galleries according to the instructions of the manufacturer (bioMérieux) and are given in the species description. Most notably, strain G1A_585^T produced the enzymes β-glucosidase and β-galactosidase and assimilates D-glucose is assimilated as sole carbon source of the tested substrates (Tab. 1).

Taken together, strain G1A_585^T is a representative of the *Sphingomonadaceae* that exhibits typical characteristics of this family. These characteristics include ubiquinone-10 as the major quinone, the polar lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidyl-dimethylethanolamine, phosphatidylcholine, sphingoglycolipids and glycolipids. The detected polyamine composition of strain G1A_585^T is characteristic for the genera *Sphingopyxis*, *Novosphingobium* and *Sphingomonas*. However, strain G1A_585^T forms a separated phylogenetic branch that clearly diverges from members of the genus *Sphingopyxis*. Distinct properties of strain G1A_585^T comprise a specific fatty acid composition, a low G+C content, the presence of β-glucosidase and β-galactosidase, and a very narrow spectrum of carbon substrates utilized for growth.

Queries of the GenBank 16S rRNA gene sequence database by BLAST (Altschul, 1997) revealed that environmental sequences identical to the 16S rRNA gene sequence of G1A_585^T have been repeatedly detected also in other freshwater lakes. Based on its distinct properties and wide distribution, we propose a novel genus and species, *Sphingorhabdus planktonica* for the novel type of bacterium.

4.3.1 Description of *Sphingorhabdus* gen. nov.

Sphingorhabdus [Sphin.go.rhab´dus. N.L. n. *sphingosinum* (from Gr. gen. n. *sphingos*, of sphinx, and suff.-*ine*), sphingosine; N.L. pref. *sphingo*-, pertaining to sphingosine; Gr. fem. n. *rhabdos*, rod; N.L. fem. n. *Sphingorhabdus*, a sphingosine-containing rod].

Cells are non-motile, gram negative, aerobic, non-spore forming rods. They contain ubiquinone-10 as major respiratory quinone and diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl- ethanolamine, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine, phosphatidylcholine, sphingoglycolipids, three glycolipids and one unknown lipid as polar lipids. As polyamines, spermidine and traces of 1,3-diaminopropane, putrescine and spermine were detected. Cells are positive for oxidase

and catalase. Major non polar fatty acids are 18:1*ω7c* and/or 18:1*ω6c* (summed feature 8) (38.19%) and 16:1*ω7c*, 16:1*ω6c* and/or 15:0 iso 2-OH (summed feature 3) (33.55%). Unusually high amounts of 2-hydroxy myristic acid (14:0 2-OH) are present (17.75%). Additional fatty acids are 16:0 (8.04%), 16:1 2-OH (1.72%), 14:0 (0.67%), 17:0 (1.46%), 16:1ω9c (0.91%), 16:0 2-OH (0.71%) and 15:0 (0.18%). The fatty acid 18:1*ω7c* 11-methyl, that is a typical constituent within *Sphingomonadaceae* species was not detected. The type species is *Sphingorhabdus planktonica*.

4.3.2 Description of *Sphingorhabdus planktonica* sp. nov.

Sphingorhabdus planktonica [plank.to´ni.ca N.L. fem. adj. *planktonica* (from Gr. adj. *planktos*, wandering), living in the plankton, planktonic].

Sphingorhabdus planktonica strain G1A_585^T (DSM 25081^T) was isolated from the freshwater lake Walchensee (near Kochel, Bavaria, Germany). General characteristics are the same as those given in the description of the genus. Colonies are yellowish-orange and transparent. Cells are 1.17 μm ± 0.20 μm long and 0.47 μm ± 0.07 μm wide. Cells absorb at 427 nm, 453 nm and 481 nm, indicating the presence of carotinoids. The G+C content of the genomic DNA is 55.7 mol%. Strain G1A_585^T is able to grow between pH 6.5 and pH 9, with optimal growth between pH 7 and 8. The temperature optimum is 18°C to 24°C. No growth detected below 11°C and above 27°C. D-glucose is utilized for growth, whereas L-arabinose, D-mannose, D-mannitol, N-acetyl-glocosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid were not utilized. Nitrates were not reduced, indole was not produced and fermentation of D-Glucose was not observed. Arginine dihydrolase, urease and protease are absent, but β-glucosidase and β-galactosidase are produced.

The type strain is G1A_585^T (DSM 25081^T).

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4.5 References

Altschul, S. F. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.

Asker, D., Beppu, T., & Ueda, K. (2007). *Sphingomonas jaspsi* sp. nov., a novel carotenoid-producing bacterium isolated from Misasa, Tottori, Japan. *Int J Syst Evol Microbiol* **57,** 1435-1441.

Balkwill, D. L., Frederickson, J. K. & Fromine, M. F. (2006). *Sphingomonas* and related genera. *Prokaryotes* **7**, 605-629.

Bartscht, K., Cypionka, H. & Overmann, J. (1999). Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. *FEMS Microbiol Ecol* **28**, 249-259.

Basta, T., Buerger, S. & Stolz, A. (2005). Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. *Microbiol* **151**, 2025-2037.

Bruns, A., Cypionka, H. & Overmann, J. (2002). Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central baltic sea. *Appl Environ Microbiol* **68**, 3978-3987.

Bruns, A., Nübel, U., Cypionka, H. & Overmann, J. (2003). Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. *Appl Environ Microbiol* **69**, 1980-1989.

Busse, H. J. & Auling, G. (1988). Polyamine patterns as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1-8.

Busse, H. J., Denner, E. B. M., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A. & Kämpfer, P. (2003). *Sphingomonas aurantiaca* sp. nov.*, Sphingomonas aerolata* sp. nov. and *Sphingomonas faeni* sp. nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas. Int J Syst Evol Microbiol* **53***,* 1253–1260.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461-466.

Chen, C., Zheng, Q., Wang, Y. N., Yan, X. J., Hao, L. K., Du, X. & Jiao, N. (2010). *Stakelama pacifica* gen. nov., sp. nov., a new member of the family *Sphingomonadaceae* isolated from the Pacific Ocean. *Int J Syst Evol Microbiol* **60**, 2857-2861.

Choi, J. H., Kim, M. S., Jung, M. J., Roh, S. W., Shin, K. S. & Bae, J. W. (2010). *Sphingopyxis soli* sp. nov., isolated from landfill soil. *Int J Syst Evol Microbiol* **60**, 1682-1686.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Denner, E. B. M., Paukner, S., Kämpfer, P., Moore, E. R. B., Abraham, W.-R., Busse, H.-J., Wanner, G. & Lubitz, W. (2001). *Sphingomonas pituitosa* sp. nov., an exopolysaccharide-producing bacterium that secretes an unusual type of sphingan. *Int J Syst Evol Microbiol* **51**, 827–841.

Gich, F. & Overmann, J. (2006). *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a novel bacteriochlorophyll a-containing, obligately aerobic bacterium isolated from freshwater lakes. *Int J Syst Evol Microbiol* **56**, 847-854.

Gich, F., Schubert, K., Bruns, A., Hoffelner, H. & Overmann, J. (2005). Specific detection, isolation and characterization of selected, previously uncultured members of freshwater bacterioplankton. *Appl Environ Microbiol* **71**, 5908-5919.

Glöckner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R. (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. *Appl Environ Microbiol* **66**, 5053-5065.

Godoy, F., Vancanneyt, M., Martinez, M., Steinbuchel, A., Swings, J. & Rehm, B. H. (2003). *Sphingopyxis chilensis* sp. nov., a chlorophenol-degrading bacterium that accumulates polyhydroxyalkanoate, and transfer of *Sphingomonas alaskensis* to *Sphingopyxis alaskensis* comb. nov. *Int J Syst Evol Microbiol* **53**, 473-477.

Jaspers, E., Nauhaus, K., Cypionka, H. & Overmann, J. (2001). Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. *FEMS Microbiol Ecol* **36**, 153-164.

Jenkins, C. L., Andrewes, A. G., McQuade, T. J. & Starr, M. P. (1979**).** The pigment of *Pseudomonas paucimobilis* is a carotenoid (nostoxanthin) rather than a brominated aryl-polyene (xanthomonadin). *Curr Microbiol* **3***,* 1–4.

Jogler, M., Siemens, H., Chen, H., Bunk, B., Sikorski, J. & Overmann, J. (2011). Identification and targeted cultivation of abundant novel freshwater sphingomonads and analysis of their population substructure. *Appl Environ Microbiol* **20**, 7355-7364.

Kalnenieks, U. (2006). Physiology of *Zymomonas mobilis*: some unanswered questions. *Adv Microb Physiol* **51**, 73-117.

Kämpfer, P., Arun, A. B., Young, C. C., Busse, H. J., Kassmannhuber, J., Rossello-Mora, R., Geueke, B., Rekha, P. D. & Chen, W. M. (2011). Proposal of *Sphingomicrobium lutaoense* gen. nov. sp. nov., isolated from a coastal hot spring of Green Island (Lutao). *Int J Syst Evol Microbiol* **ahead of print**.

Kim, M. K., Schubert, K., Im, W.-T., Kim, K.-H., Lee, S.-T. & Overmann, J. (2007). *Sphingomonas kaistensis* sp. nov., a novel alphaproteobacterium containing *pufLM* genes. *Int J Syst Evol Microbiol* **57**, 1527-1534.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115-175. Edited by E. Stackebrandt & M. Goodfellow. Chichester, UK: Wiley.

Lee, M., Ten, L. N., Lee, H.-W., Oh, H. W., Im, W.-T. & Lee, S.-T. (2008) *Sphingopyxis ginsengisoli* sp. nov., isolated from soil of a ginseng field in South Korea. *Int J Syst Evol Microbiol* **58,** 2342-2347.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363-1371.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Evol Microbiol* **39**, 159–167.

Sharma, P., Verma, M., Bala, K., Nigam, A. & Lal, R. (2010). *Sphingopyxis ummariensis* sp. nov., isolated from a hexachlorocyclohexane dump site. *Int J Syst Evol Microbiol* **60**, 780-784.

Sikorski, J., Brambilla, E., Kroppenstedt, R. M. & Tindall, B. J. (2008). The temperature-adaptive fatty acid content in *Bacillus simplex* strains from 'Evolution Canyon', Israel. *Microbiol* **154**, 2416-2426.

Srinivasan, S., Kim, M. K., Sathiyaraj, G., Veena, V., Mahalakshmi, M., Kalaiselvi, S., Kim, Y. J. & Yang, D. C. (2010). *Sphingopyxis panaciterrulae* sp. nov., isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* **60**, 2358-2363.

Stead, D. E., Sellwood, J. E., Wilson, J. & Viney, I. (1992). Evaluation of a commercial microbial identification system based on fatty acid profiles for rapid, accurate identification of plant pathogenic bacteria. *J Appl Bacteriol* **72**, 315-321.

Stolz, A., Schmidt-Maag, C., Denner, E. B. M., Busse, H.-J., Egli, T. & Kämpfer, P. (2000). Description of *Sphingomonas xenophaga* sp. nov. for strain BN6^T and N,N, which degrade xenobiotic aromatic compounds. *Int J Syst Evol Microbiol* **50***,* 35–41.

Stolz, A., Busse, H.-J. & Kämpfer, P. (2007). *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57,** 572–576.

Takeuchi, M., Hamana, K. & Hiraishi, A. (2001). Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. *Int J Syst Evol Microbiol* **51**, 1405-1417.

Takeuchi, M., Kawai, F., Shimada, Y. & Tokota, A. (1993) Taxonomic Study of Polyethylene Glycol-Utilizing Bacteria: Emended Description of the Genus *Sphingomonas* and New Descriptions of *Sphingomonas macrogoltabidus* sp. nov., *Sphingomonas sanguis* sp. nov. and *Sphingomonas terrae* sp. nov. *System Appl Microbiol* **16**, 227-238

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Letters* **25**, 125-128.

Tindall, B. J. (1990a). Lipid composition of *Halobacterium lacusprofundi*. *FEMS microbiol lett* **66**, 199–202.

Tindall, B. J. (1990b). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Vancanneyt, M., Schut, F., Snauwaert, C., Goris, J., Swings, J. & Gottschal, J. C. (2001). *Sphingomonas alaskensis* sp. nov., a dominant bacterium from a marine oligotrophic environment. *Int J Syst Evol Microbiol* **51**, 73-79.

Wittich, R. M., Wilkes, H., Sinnwell, V., Francke, W. & Fortnagel, P. (1992). Metabolism of dibenzo-p-dioxin by *Sphingomonas sp.* strain RW1. *Appl Environ Microbiol* **58**, 1005-1010.

Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T. & Yamamoto, H. (1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* **34**, 99-119.

Yoon, J. H. & Oh, T. K. (2005). *Sphingopyxis flavimaris* sp. nov., isolated from sea water of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**, 369-373.

Yoon, J. H., Lee, C. H., Yeo, S. H. & Oh, T. K. (2005). *Sphingopyxis baekryungensis* sp. nov., an orange-pigmented bacterium isolated from sea water of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**, 1223-1227.

Zhang, D. C., Liu, H. C., Xin, Y. H., Zhou, Y. G., Schinner, F. & Margesin, R. (2010). *Sphingopyxis bauzanensis* sp. nov., a psychrophilic bacterium isolated from soil. *Int J Syst Evol Microbiol* **60**, 2618-2622.

Zwart, G., Crump, B. C., Kamst-Van Agterveld, M. P., Hagen, F. & Han, S. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**, 141-155.

4.6 Supplementary figures

Figure S1 A. Phase-contrast photomicrographs, **B.** transmission electron micrographs and **C.** scanning electron micrographs of cells of strain G1A_585^T.

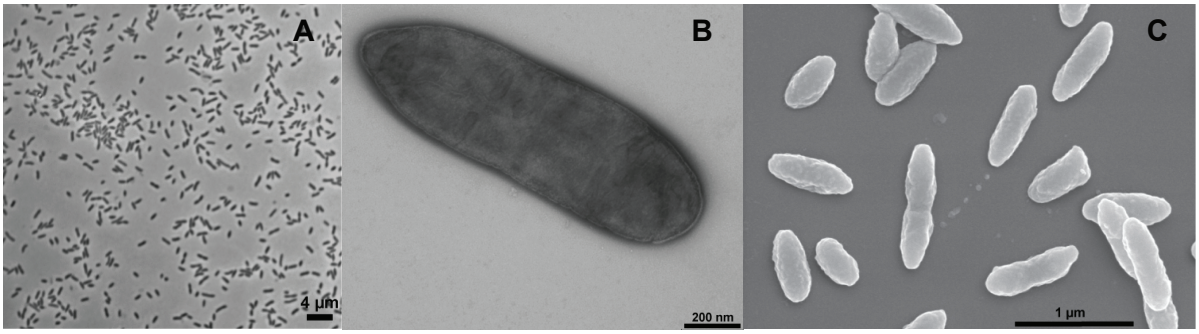
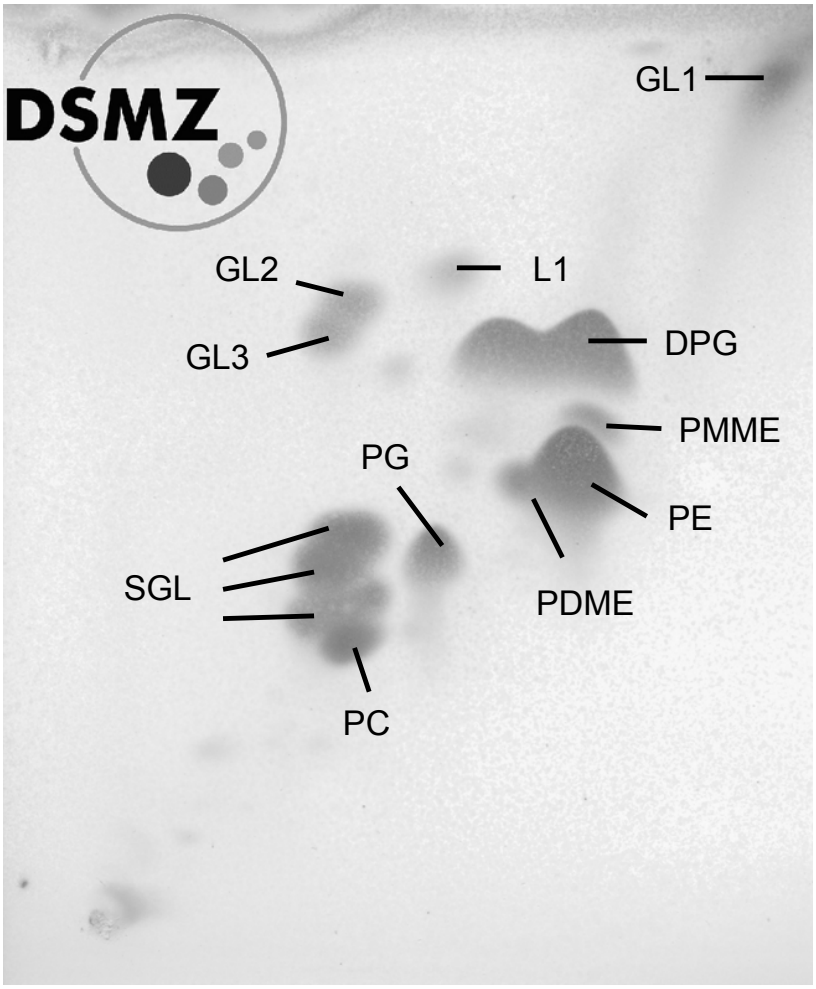


Figure S2. Polar lipid profile of strain G1A_585^T after separation with two-dimensional thin layer chromatography. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; PC, phosphatidylcholine; SGL, sphingoglycolipids; L1, unidentified lipid; GL1- GL3, glycolipids.



4.7 Supplementary tables

Supplementary Table S1. Cellular fatty acid profile of strain G1A_585^T and valid described *Sphingopyxis* species.

1, G1A_585^T (this study); 2, *S. terrae* IFO 15098^T (Sharma *et al.*, 2010); 3, *S. alaskensis* DSM 13593^T (Vancanneyt *et al.*, 2001); 4, *S. ummariensis* DSM 24316^T (Sharma *et al.*, 2010); 5, *S. macrogoltabida* 8826^T (this study); 6, *S. witflariensis* DSM 14551^T; 7, *S. ginsengisoli* LMG 23390^T (Srinivasan *et al.*, 2010); 8, *S. baekryungensis* DSM 16222^T (Yoon *et al.*, 2005); 9, *S. flavimaris* DSM 16223^T (Yoon & Oh, 2005); 10, *S. bauzanensis* DSM 22271^T (Zhang *et al.*, 2010); 11, *S. soli* KCTC 22405^T (Choi *et al.*, 2010); 12, *S. taejonensis* KCTC 2884^T (Srinivasan *et al.*, 2010); 13, *S. chilensis* DSM 14889^T (Godoy *et al.*, 2003); 14, *S. panaciterrulae* KCTC 22112^T (Srinivasan *et al.*, 2010); tr, >1%.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
non polar fatty acids														
14:0	tr	-	tr	-	tr	0.1	2.1	1.2	-	-	-	-	tr	1.5
15:0	tr	-	2.8 (±0.2)[1]	-	tr	2.9	1.7	2.6	1.5	-	3.9	3.1	tr	2.1
16:0	8.0	4.3	7.3 (±0.7)[1]	14.4	6	2.1	10.5	9.7	9.1	8.2	4.7	22.7	9	15.6
17:0	1.5	4.1	2.7 (±0.6)[1]	-	-	tr	4.1	3.9	2.1	-	4.5	2.4	1	2.3
17:0 cyclo	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18:0	-	-	-	2.4	tr	tr	-	-	-	tr	-	tr	-	tr
15:1 *ω6c*	-	-	-	-	-	tr	-	-	1.3	-	-	-	-	-
16:1 *ω5c*	-	tr	1.1 (±0.2)[1]	3.3	1.9	1.3	-	-	-	2.8	1.2	2.3	1	-
16:1 *ω9c*	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-
17:1 *ω6c*	1.1	43.5	33.2 (±3.4)[1]	9.6	0.8	22.1	17	18.8	17.8	5.3	44.2	10.5	18	7.3
17:1 *ω8c*	-	7.5	7.6 (±1.0)[1]	-	-	4.2	3.1	2.4	3.2	-	6.9	1.5	3	2.2
17:1 iso *ω9c*	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-
18:1 *ω5c*	-	1	tr		tr	tr			1.7	tr	tr	-	tr	1.5
18:1 *ω7c* 11-methyl	-	1.1	1.7 (±0.2)[1]	10.9	5.03	6.2	8.1	5.6	4.5	3.1	2	tr	2	tr
Hydroxy fatty acids														
13:0 2-OH	-	-	-	-	-	tr	-	-	-	-	-	-	-	-
14:0 2-OH	17.8	1.6	1.3 (±0.3)[1]	6.1	1.9	3.1	9.7	8.9	2.8	8	1.3	4.3	4	tr
15:0 2-OH	-	11.8	4.6 (±0.8)[1]	4.1	-	8.4	5.2	4.2	6.1	1.7	14.7	2.3	4	3.2
16:0 2-OH	tr	1.2	1.4 (±0.4)[1]	3.2	1.19	2.4	2.3	1.1	4.9	5	1.1	1.5	2	
16:1 2-OH	1.7	-	-	-	-	tr	-	-	-	-	-	-	-	
16:0 iso 3-OH	-	-	-	-	0.27	-	-	-	-	-	-	-	-	
18:1 2-OH	-	-	-	-	0.11	tr	-	-	-	-	-	-	-	
Summed features∗														

3	30.6	6.7	8.2 (±1.4)[1]	14.7	34.8	23.3	6.2	-	20.3	37.4	2.9	17.4	18	18.6
7	-	-	-	-	-	tr	-	-	-	-	-	6.4	-	15.4
8	38.2	11	26.5 (±2.6)[1]	28.3	47.2	25.0	27.8	33.8	24	19.6	10.6	23.6	34	27.5

*Summed feature 3 consists of 16:1*ω7c*, 16:1*ω6c* and/or 15:0 iso 2-OH; summed feature 7 contained un18.846, 19:1 *ω6c* and/or 19cy; summed feature 8 included 18:1*ω7c* and/or 18:1*ω6c*.
[1] summarized data from 7 strains.

Chapter 5

Large scale distribution and activity patterns of an extremely low-light adapted population of green sulfur bacteria in the Black Sea

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5.1 Contribution of the author

Mareike Jogler established the DNA and RNA extraction protocols for concentrated Black Sea water samples and performed the ITS1 qPCR from these DNA and RNA samples. Mareike Jogler together with Jörg Overmann provided the corresponding figure 5, experimental procedure, results and discussion sections. During Mareike Joglers Diplomathesis, she generated the data for the ATP measurements and provided the corresponding figure 7 as well as the experimental procedure, results and discussion sections together with Jörg Overmann.

5.2 Abstract

The Black Sea chemocline represents the largest extant habitat of anoxygenic phototrophic bacteria and harbours a monospecific population of *Chlorobium* phylotype BS-1. High sensitivity measurements of underwater irradiance and sulfide revealed that the optical properties of the overlying water column were similar across the Black Sea basin, whereas the vertical profiles of sulfide varied strongly between sampling sites and caused a dome-shaped three-dimensional distribution of the green sulfur bacteria. In the centres of the western and eastern basins the population of BS-1 reached upward to depths of 80 and 95 m, respectively, but were detected only at 145 m depth close to the shelf. Using highly concentrated chemocline samples from the centres of the western and eastern basins, the cells were found to be capable of anoxygenic photosynthesis under *in situ* light conditions and exhibited a photosynthesis-irradiance curve similar to low-light adapted laboratory cultures of *Chlorobium* BS-1. Application of a highly specific RT-qPCR method which targets the internal transcribed spacer (ITS) region of the *rrn* operon of BS-1 demonstrated that only cells at the central station are physiologically active in contrast to those at the Black Sea periphery. Based on the detection of ITS-DNA sequences in the flocculent surface layer of deep sea sediments across the Black Sea, the population of BS-1 has occupied the major part of the basin for the last decade. The continued presence of intact but non-growing BS-1 cells at the periphery of the Black Sea indicates that the cells can survive long-distant transport and exhibit unusually low maintenance energy requirements. According to laboratory measurements, *Chlorobium* BS-1 has a maintenance energy requirement of ~1.6-4.9·10^{-15} kJ·$cell^{-1}$·d^{-1} which is the lowest value determined for any bacterial culture so far. *Chlorobium* BS-1 thus is particularly well adapted to survival under the extreme low-light conditions of the Black Sea, and can be used as a laboratory model to elucidate general cellular mechanisms of long-term starvation survival. Because of its adaptation to extreme low light marine environments, *Chlorobium* BS-1 also represents a suitable indicator for palaeoceanography studies of deep photic zone anoxia in ancient oceans.

5.3 Introduction

The Black Sea represents the largest extant anoxic water body on Earth. Its oxic-anoxic transition zone is positioned at about 100 m depth and characterised by an intense cycling of sulfur and carbon, as reflected by elevated rates of sulfide oxidation (Jørgensen *et al.*, 1991), sulfate reduction and of dark CO_2-fixation (Pimenov *et al.*, 2000). Correspondingly, maxima of sulfate reducing bacteria (Neretin *et al.*, 2007) and *Epsilonproteobacteria*, which comprise many microaerophilic sulfur oxidisers (Lin *et al.*, 2006), have been found in the transition zone. In addition, bacteriochlorophyll *e* (BChl *e*) has repeatedly been detected in the chemocline of the Black Sea (Repeta *et al.*, 1989; Coble *et al.*, 1991; Gorlenko *et al.*, 2005; Manske *et al.*, 2005; Koblizek *et al.*, 2006; Overmann & Manske, 2006) indicating the presence of green sulfur bacteria (*Chlorobiaceae*).

A single and novel 16S rRNA gene type of *Chlorobiaceae* was identified by culture-independent methods in the chemocline samples (phylotype BS-1; Manske *et al.*, 2005). This phylotype thus far is unique for the Black Sea and could be enriched in laboratory cultures. Cultures were shown to be capable of exploiting very low light intensities for photosynthetic CO_2 fixation (Manske *et al.*, 2005). Similar to all known green sulfur bacteria which are obligate anoxygenic photolithoautotrophs (Pfennig & Trüper, 1989; Overmann, 2001), culture experiments, as well as $\delta^{13}C$ values of specific biomarkers isolated from the natural population, indicated that BS-1 grows autotrophically *in situ* (Overmann *et al.*, 1992; Manske *et al.*, 2005). Yet, measurements of downwelling irradiance in the chemocline revealed maximum values of ≤ 0.0022 μmol quanta$\cdot m^{-2}\cdot s^{-1}$ (Manske *et al.*, 2005) which represent by far the lowest values reported for any habitat of photosynthetic organisms. Repeated attempts to demonstrate anoxygenic photosynthetic activity directly in chemocline samples have so far been unsuccessful (Jørgensen *et al.*, 1991; Glaeser *et al.*, 2003).

The horizontal extent of the monospecific assemblage of green sulfur bacteria in the Black Sea is unknown since previous investigations have focused on only one or two sampling locations. In the Black Sea, the oxic-anoxic interface is dome shaped due to strong rim currents which occur at the periphery of the basin and vertically displace the chemocline towards greater depths (Oguz *et al.*, 1994). Thus, the sulfidic zone extends upward to 82-110 m below sea surface in the Black Sea centre, but reaches depths of 150-180 m at its periphery (Codispoti *et al.*, 1991; Sorokin, 2002; Manske *et al.*, 2005). Yet, BChl *e* was present at a station close to the Black Sea shelf (Manske *et al.*, 2005). It therefore appears possible that a considerable fraction of the BS-1 population consists of inactive or moribund cells. Initial growth experiments indicated an unusually low maintenance energy requirement of the Black

Sea phylotype compared to other green sulfur bacteria or other anoxygenic or oxygenic phototrophs (Overmann *et al.*, 1992). However, no quantitative estimate of this parameter could so far be obtained for *Chlorobium* BS-1.

Up to 6,000 year-old sediment layers of the Black Sea harbour the carotenoid isorenieratene which is almost exclusively found in green sulfur bacteria (Sinninghe Damsté *et al.*, 1993). Hence, green sulfur bacteria may have already been present in the Black Sea during earlier stages. While BS-1 represents the only extant green sulfur bacterial phylotype which is currently found in the chemocline, a recent analysis of sediments at two sampling locations in the western basin of the Black Sea recovered a considerable diversity of 14 different *Chlorobiaceae* 16S rRNA gene sequence types, but only traces of the BS-1 phylotype in few of the sediment layers (Manske *et al.*, 2008). In order to more precisely reconstruct the structure of past green sulfur bacterial communities in the Black Sea (and similar marine environments like the ancient Eastern Mediterranean Sea; Coolen & Overmann, 2007), the correlation between the chemocline biomass of BS-1 and its deposition in the deep-sea sediments needs to be elucidated.

The aim of the present study was to gain a more solid understanding of the factors determining the population dynamics of phylotype BS-1 in the chemocline and its imprint in the deep sea sediments. To this end, horizontal variations in the environmental conditions and the population density of phylotype BS-1, the photosynthetic activity and maintenance energy requirement of the natural BS-1 population, as well as the deposition of BS-1 at the surface of deep-sea sediments were analyzed at sampling locations across the Black Sea.

5.4 Results and Discussion

5.4.1 Distribution of green sulfur bacteria and its environmental determinants

Green sulfur bacteria are obligately anoxygenic photolithoautotrophs and require the simultaneous presence of light and sulfide for growth. So far, the horizontal variation of the underwater light climate in the Black Sea was not known. Therefore, vertical gradients of both environmental factors were analyzed at seven different main stations.

Downwelling irradiance decreased exponentially with depth (Fig. 2). The decrease was steeper in the upper 40 m of the water column, which has to be attributed to the successive narrowing of the underwater light spectrum with depth (Jewson *et al.*, 1984). Such a spectral narrowing has previously been documented for the upper water layers of the Black Sea (Dr. Falk Pollehne, Institute of Baltic Sea Research Warnemünde, pers. comm.). For water layers below 40 m, the vertical attenuation coefficient k_D was very similar between sampling stations. The average value for k_D calculated for all 7 stations was (0.0844 ± 0.0021) m^{-1}, indicating that the optical properties of the oxic water layers and the anoxic layers above the population of green sulfur bacteria were similar across the Black Sea basin.

In contrast to downwelling irradiance, the vertical profiles of sulfide concentrations varied strongly between sampling locations (Fig. 2). In the centres of the western and eastern basins, sulfide was already detectable at a depth of 95 m (station 5) or 110 m (station 21), respectively. In contrast, sulfide was detected only beneath 160 m depth at the station closest to the northern shelf (station 13) (Fig. 2). After a rise of the chemocline by 30 m between the years 1969 and 1988 (Murray *et al.*, 1989), the upper limit of the sulfidic zone had reached a water depth of 95 m in the centre of the Black Sea basin already in 1988. Obviously, the vertical position of the chemocline has stabilised and no major changes in the hydrographic structure have occurred over the last two decades.

The potential density σ_θ of the topmost sulfidic water layers was similar at the different sampling stations and ranged between 16.1 and 16.2 $kg \cdot m^{-3}$ (Fig. 1B). The mean of σ_θ = 16.15 $kg \cdot m^{-3}$ corresponds to the value determined in recent years (Yakushev *et al.*, 2006) as well as a decade ago (Murray *et al.*, 1995). Employing the potential density of 16.15 $kg \cdot m^{-3}$ as an indicator for the top of the sulfidic zone, the variations in the vertical position of the sulfidic zone were analyzed across the Black Sea basin. The resulting three-dimensional pattern (Fig. 1A) clearly reflects the circulation pattern in the Black Sea which features cyclonic currents in the centre of the western and eastern basins and several smaller anticyclonic gyres at the periphery, which together cause a dual dome-shaped structure of the

upper Black Sea water body (Oguz *et al.*, 1992, 1993). While the upper position of the sulfidic zone varied considerably, the slope of the individual vertical sulfide profiles and hence the upward diffusive flux of sulfide into the chemocline was comparable between the different stations. In conclusion, the light which is available to green sulfur bacteria in the Black Sea is largely determined by the depth of the sulfidic zone and hence the hydrographic structure of the environment.

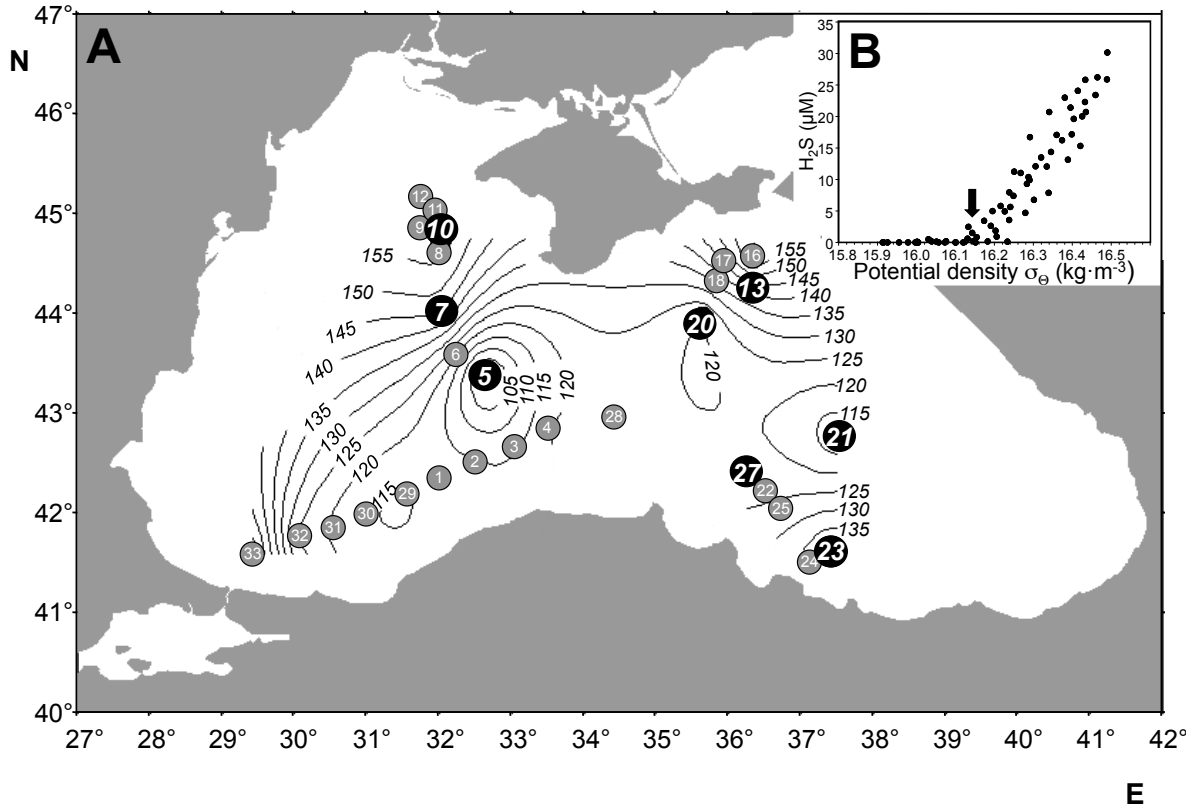

Figure 1A. Map of the Black Sea with main sampling stations (black points) and stations for the determination of a limited number of parameters in the chemocline (grey points). Numbers of the stations are provided (compare SUppl. Table 1). Isopleths depict vertical position of water layer with a potential density σ_θ = 16.15 kg·m^{-3} which corresponds to the top of the sulfidic monimolimnion (water depths at isopleths given in m). **B.** Correlation of sulfide concentrations and potential density of Black Sea water. *Arrow* indicates minimum density (σ_θ = 16.15 kg·m^{-3}) of sulfidic Black Sea water. For clarity, some of the sampling locations in the vicinity of S13 were omitted (compare Fig. 6).

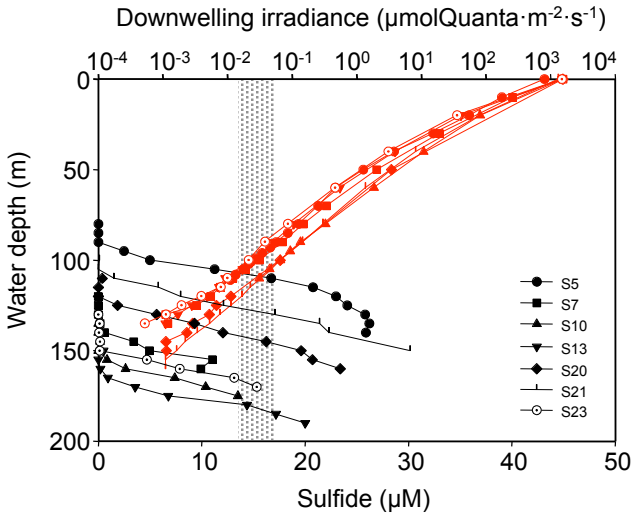

Figure 2. Vertical profiles of downwelling irradiance (red symbols and lines) and sulfide concentrations at 7 sampling locations across the Black sea. Stippled area marks the threshold for photosynthetic carbon fixation (between 0.015 and 0.055 µmol quanta·m^{-2}·s^{-1}) as determined in the natural population and laboratory cultures of *Chlorobium* BS-1.

As in previous studies, (Overmann *et al.*, 1992; Manske *et al.*, 2005), bacteriochlorophyll *e* (BChl*e*) and isorenieratene (Suppl.Fig. 1) were the only photosynthetic pigments detected in the chemocline of the Black Sea employing our sensitive HPLC technique. The dominant Bchl*e* homologs were geraniol (BChle_G) and farnesol (BChle_F) esters. However, the relative composition of the homologs differed between the sampling locations. With increasing depth of the chemocline, the predominant ethyl/ethyl [E,E]-BChle_G and ethyl/methyl [E,M]-BChle_F were replaced by homologs containing longer aliphatic side chains, specifically propyl/ethyl [P,E]-BChle_G, isobutyl/ethyl [I,E]-BChl e_G, and [E,E]-BChle_F, [P,E]-BChle_F, [I,E]-BChle_F. The relative amounts of secondary homologs ([E,E]-Bchle_{HEN} and isobutyl/ethyl-BChle_{HEN}, esterified with hexadecenal) increased concomitantly (Suppl.Fig. 1). Pigments of other green sulfur bacteria (BChl*c*, *d* or chlorobactene) or of purple sulfur bacteria (e.g. spirilloxanthin, okenone) were not detected.

The population of BS-1 extended over a depth interval of about 40 m at all stations. Highest concentrations of BChl*e* were determined at station no. 5 and reached 600 ng BChl*e*·l^{-1} at 85 m depth (Suppl.Fig. 2). At stations no. 21 and 23, maximum concentrations were 220 and 160 ng BChl*e*·l^{-1} and were observed at a water depth of 105 and 135 m, respectively. Peak BChl*e* values at the remaining stations were low and very similar, ranging between 58 and 73 ng BChl*e*·l^{-1}. Because of the uniform light distribution in the Black Sea, the depth of the Bchl*e* maximum was mainly determined by the depth of the upper limit of sulfide-containing water layers (Fig. 3A). At stations S5 and S21, maximum BChl*e*

concentrations were detected above sulfide-containing water layers whereas the two parameters matched at all the other stations. Therefore, part of the population of BS-1 at the two central stations occurred at sulfide concentrations below 0.2 µM.

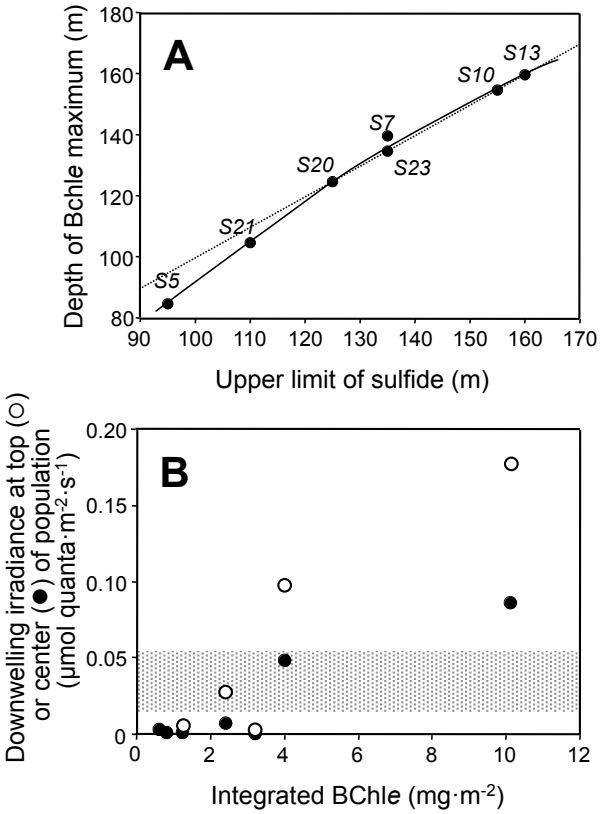

Figure 3A. Correlation between the depth of the Bchl*e* maximum and the upper limit of sulfide-containing water layers at seven main sampling stations. The detection limit of sulfide was 0.2 µM. The dotted line indicates equality for both parameters. **B.** Relationship between the integrated amount of BChl*e* and the downwelling irradiance reaching the water layer of the top of the BS-1 population (○) or the Bchl*e* maximum (●) at seven main sampling stations. Stippled area marks the threshold for photosynthetic carbon fixation (between 0.015 and 0.055 µmol quanta·m^{-2}·s^{-1}) as determined in the natural population and laboratory cultures of *Chlorobium* BS-1.

In low-light adapted laboratory cultures, phylotype BS-1 reaches light saturation of photosynthesis and of growth above a light quantum flux of 1 µmol quanta·m^{-2}·s^{-1} (Overmann *et al.*, 1992; Manske *et al.*, 2005). In the Black Sea, downwelling irradiance never exceeded 0.18 µmol quanta·m^{-2}·s^{-1} even at the shallowest station and at the top of the population of green sulfur bacteria (Fig. 2, white circles in Fig. 3B). Since our measurements were conducted around solar noon, close to the summer solstice and after the spring diatom bloom (Yilmaz *et al.*, 2006), it has to be concluded that green sulfur bacteria will never attain light saturation of photosynthetic growth anywhere in the Black Sea chemocline. Given the significantly deeper localisation of the chemocline at the periphery of the Black Sea, part of

the population of BS-1 may even not be photosynthetically active at all. The observation that sulfide concentrations in the major part of the BS-1 population were decreased much more in the centres of the western and eastern basins than at the periphery also suggests considerable horizontal differences in photosynthetic activity. It was therefore investigated whether the available irradiance can support growth of *Chlorobium* BS-1 at all locations where these bacteria occur in the chemocline.

5.4.2 Horizontal variations in physiological activity of *Chlorobium* BS-1 across the Black Sea basin and other determinants of biomass distribution

Previous attempts to demonstrate anoxygenic photosynthetic activity in chemocline water samples have been unsuccessful (Jørgensen *et al.*, 1991; Glaeser *et al.*, 2003). However, when cells were concentrated 500 to 1100-fold by *in situ* pumps in the present investigation, light-dependent incorporation of $H^{14}CO_3^-$ was detected at 0.149 and 0.055 µmol quanta·m^{-2}·s^{-1} but not at 0.0033 µmol quanta·m^{-2}·s^{-1} (Fig. 4). Laboratory cultures of *Chlorobium* BS-1 have been shown to be photosynthetically active at ≥ 0.015 µmol quanta·m^{-2}·s^{-1} (Manske *et al.*, 2005) which is in line with the photosynthesis-irradiance curve derived for the natural population (Fig. 4). Similar to laboratory cultures, the natural population reached light saturation of photosynthesis at light intensities of about 1 µmol quanta·m^{-2}·s^{-1}.

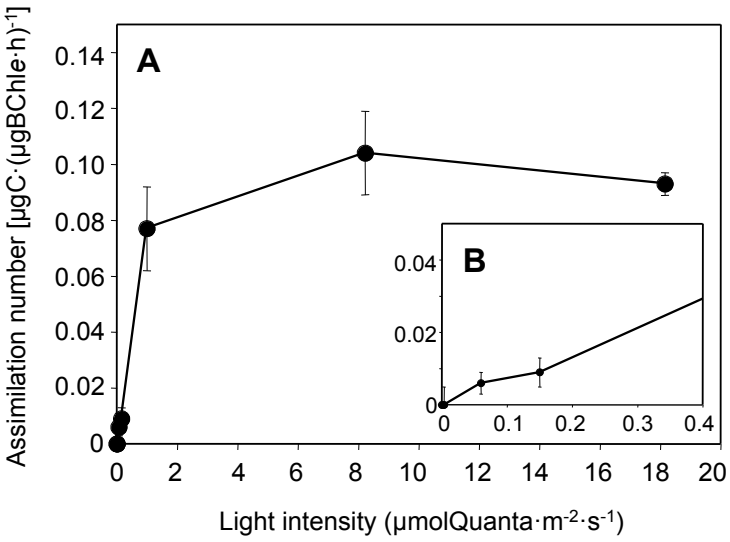

Figure 4. Light dependence of $H^{14}CO_3^-$ assimilation in chemocline samples from station 21. **A.** Assimilation numbers at light intensities between 0 and 20 µmol quanta·m^{-2}·s^{-1}. Bars represent one standard deviation. **B.** Expanded view of curves below 0.4 µmol quanta·m^{-2}·s^{-1}. Asterisks denote values which were significantly higher than dark controls ($p \leq 0.1$). Results obtained with chemocline samples from station 5 were comparable (data not shown).

At the two central stations (no. 5 and 21), the top of the BS-1 population received maximum downwelling irradiances of ≥ 0.10 µmol quanta·m^{-2}·s^{-1} due to the shallow position of the chemocline. In contrast, underwater irradiance at the other sampling positions ranged at or below the threshold for photosynthetic carbon fixation (between 0.015 and 0.055 µmol quanta·m^{-2}·s^{-1}; see above) (Fig. 3B; stippled). Consequently, a build up of *Chlorobium* BS-1 biomass by photosynthetic growth is only possible in the centre of the Black Sea basin and during summertime. Nevertheless, the population of BS-1 extended across the major part of the Black Sea basin, indicating that the cells were in a nongrowing state in most areas.

Since green sulfur bacteria maintain a constant and maximum intracellular concentration of photosynthetic pigments during light limited growth (Montesinos *et al.*, 1983), BChl*e* can serve as an indicator of green sulfur bacterial biomass in the Black Sea. At the highest *in situ* light intensities (station 5), the chemocline harboured an integrated amount of 10.1 mgBChl*e*·m^{-2}, whereas 4.0 mgBChl*e*·m^{-2} were determined at the deeper position of the chemocline at station 21. In our study, the values at the remaining stations ranged between 3.2 and 1.0 mgBChl*e*·m^{-2}, but were not correlated with available light intensity (Fig. 3B). Even at the lowermost underwater irradiance in winter time (daily maxima of ≤ 0.0022 µmol quanta·m^{-2}·s^{-1}) which are not sufficient to support photosynthetic growth, the biomass of *Chlorobium* BS-1 still amounted to 0.8 mgBChl*e*·m^{-2} (Manske *et al.*, 2005), indicating that the large scale distribution of the population of BS-1 in the Black Sea is not only determined by photosynthetic growth but additional processes.

In order to assess the physiological state of the BS-1 cells by an independent method, the concentrations of the internal transcribed spacer (ITS)-RNA of *Chlorobium* BS-1 were quantified employing a highly specific RT-qPCR method. The concentration of ITS transcripts has previously been shown to reflect the activity of bacterial cells (Schmid *et al.*, 2001). Biomass was collected by *in situ* pumps at up to 5 depths at stations no. 5, 10 and 21, the RNA extracted and the copy numbers of ITS-RNA were determined. For comparison, genomic DNA was extracted from parallel samples and used to determine the copy number of *rrn* operons at the same depth by qPCR. To account for biomass differences and variations in extraction efficiency between parallel subsamples, copy numbers of ITS transcripts and ITS-DNA were expressed in % of bacterial rRNA or % of bacterial 16S rRNA genes, respectively (Fig. 5).

In agreement with the inferred pattern of photosynthetic activity, ITS-RNA could only be detected at the central stations 5 and 21, which are located in the central western and eastern basin of the Black Sea (Fig. 5B) while no transcripts were present in cells from the 145 m

deep chemocline close to the northwestern shelf (station 10). While active cells were also detected 60 m below the chemocline in the centres, no ITS-RNA could be detected below. In contrast, ITS-DNA was present in the deepest water layer investigated (400 m) (Fig. 5A). Based on these molecular data, a considerable fraction of the *Chlorobium* BS-1 population sinks towards the sea bottom. During this process the cells become inactive over the first tens of meters. Since ITS-DNA could also be detected in oxic water layers above the chemocline at station 21 (Fig. 5A), some of the BS-1 cells must be carried into upper, oxic water layers in the centre of the Black Sea basins.

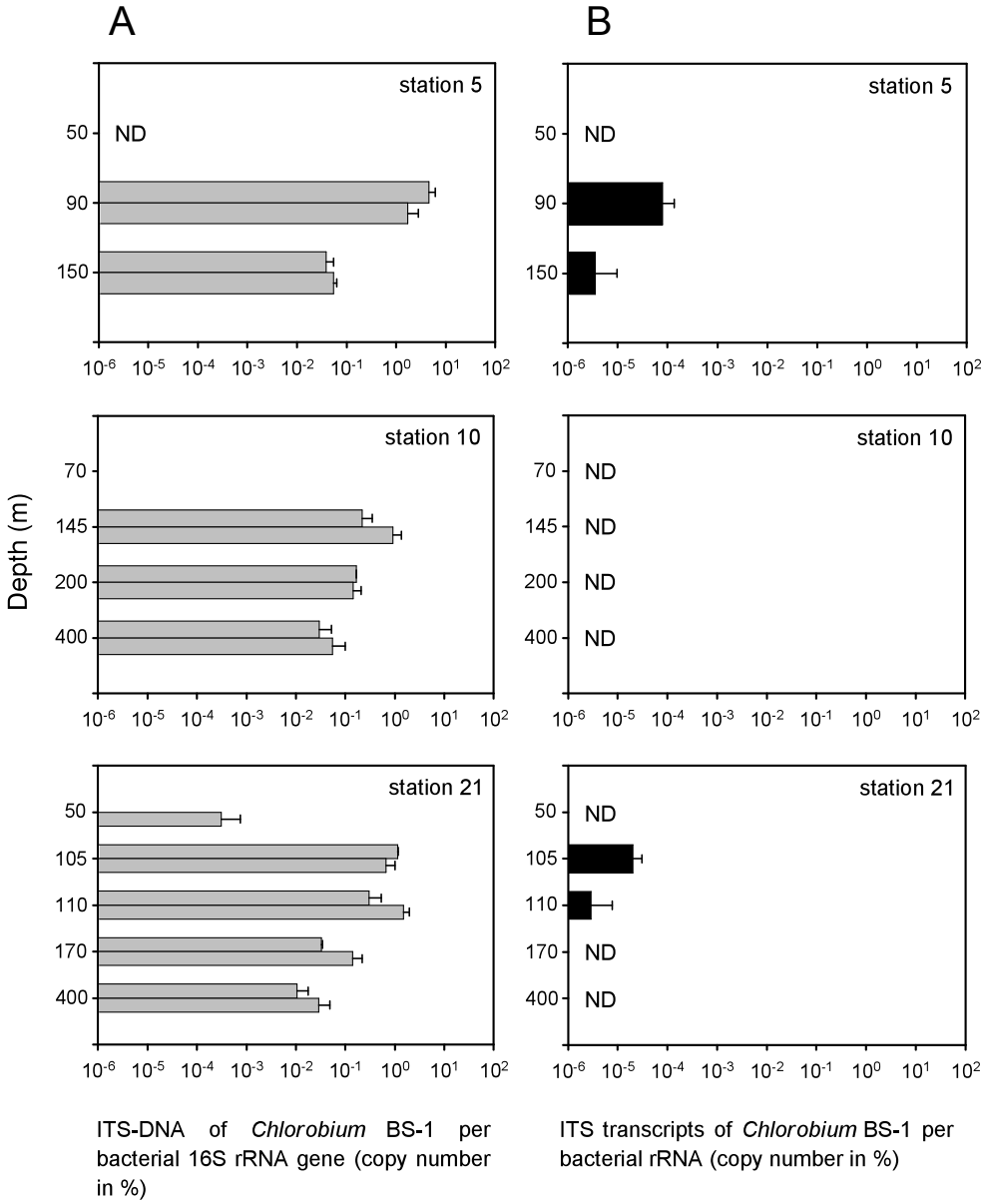

Figure 5. Quantification of internal transcribed spacer sequences of the BS1 *rrn* operon in the chemocline of the Black Sea. **A.** Ratios of BS1-ITS DNA to bacterial 16S rRNA genes in the DNA extracts. For each depth, ratios were determined separately for two different filters. **B.** Ratios of BS1-ITS transcripts to 16S rRNA in the RNA extracts. *ND* = not detected. Detection limits were 10^{-4} % for ITS DNA sequences of BS-1, and 10^{-6} % for ITS transcripts of BS-1.

Theoretically, the presence of a considerable population of *Chlorobium* BS-1 at the periphery could be caused by short-term vertical oscillations of the chemocline permitting intermittent photosynthetic growth before cells would by displaced again to greater depths. Short-term (4 h per day) periodic increases of available irradiances such as they occur during internal seiches in lakes have been shown to result in a slow build up of a bloom of green sulfur bacteria and its maintenance at the very low light intensitities of greater depths (Bergstein *et al.*, 1979). However, recent high resolution *in situ* measurements of sulfide concentrations over a three-week time interval did not yield evidence for vertical oscillations of the sulfidic layer even during the pronounced decrease of physical mixing and concomitant shoaling of the oxygen containing zone that occurs during the shift from winter to spring (Glazer *et al.*, 2006). Similarly, extended time series of sulfide measurements in the eastern Black Sea demonstrated only minor (i.e., < 10 m) seasonal and interannual changes in the position of the sulfidic zone over the past 8 years (Yakushev *et al.*, 2006). Based on the vertical gradients in underwater irradiances determined in the present study, an upward movement of the sulfidic layers by less than 10 m would still not enable photosynthetic growth outside the centre of the Black Sea (compare Fig. 2). Taken together, our physiological measurements, transcript analyses and the missing correlation between biomass distribution and underwater light field consistently indicate that the distribution of *Chlorobium* BS-1 outside the centres of the cyclonic gyres as well as its presence during winter is governed by processes other than photosynthetic growth.

At the present stage of knowledge, annually recurring horizontal water movements above the sulfidic water layers are the most plausible mechanism underlying the horizontal dispersal of *Chlorobium* BS-1 biomass in the Black Sea. During winter, a water layer exhibiting a minimum temperature (the cold intermediate layer, CIL) forms over the dome-shaped centres of the cyclonic gyres and spreads towards the periphery of the Black Sea (Oguz & Besiktepe 1999). Since genomic DNA of BS-1 could indeed be detected by our highly specific qPCR in the oxic water layers well above the chemocline in the centre of the eastern basin (Fig. 5, station 21), the CIL may entrain the upper portion of the *Chlorobium* BS-1 population and disperse the cells towards the margins of the Black Sea basin. Thus the peripheral part of the extant *Chlorobium* BS-1 population in the Black Sea likely is of allochthonous origin.

In order to elucidate whether the large nongrowing fraction of the BS-1 population is a rather short lived or a regular phenomenon, we quantified molecular remains in the topmost sediment layers across the Black Sea.

5.4.3 Molecular imprint of green sulfur bacteria in deep sea sediments

No bacteriochlorophyll *e* could be detected in sediment traps during earlier investigations (Repeta & Simpson 1991). It had therefore remained unclear, whether cells of *Chlorobium* BS-1 sediment out of the chemocline or whether cell lysis is the only quantitative loss process in the Black Sea chemocline. The sensitive and specific detection technique for ITS-DNA developed in the present work provides the first direct evidence for the sedimentation of BS-1 cells towards the Black Sea bottom, since ITS-DNA of BS-1 could be detected in particles collected at 400 m depth. In order to determine whether dispersal of BS-1 towards the Black Sea periphery represented a short term event or occurs over longer time intervals, we assessed the presence of BS-1 ITS-sequences in the flocculent surface (fluff) layer of deep-sea sediments at 19 different stations. The fluff layer integrates a time period of 3 to 14 years based on the sedimentation rates (Gulin 2000) and depending on the location.

The dry weight content of the samples ranged between 3 % and 13% (w/v), which are typical values for freshly deposited sediment matter. In contrast to the horizontal differences in the chemocline biomass of *Chlorobium* BS-1, the genomic DNA in the fluff layer varied only slightly between 18 of the 21 stations (Fig. 6). The mean content at these stations (excluding station 12, 16, 25) was determined to be 5.5 ng BS-1 DNA·(g sediment dry weight)$^{-1}$, equivalent to 0.014 % of the total genomic DNA in the sediment. Unexpectedly, a much higher concentration of 53.4 ng BS-1 DNA·(g sediment dry weight)$^{-1}$ was detected at station 25 located on the Arkhangelski Ridge. Results for the two very shallow stations 12 and 16 (water depth < 80 m) differed significantly from all others. The genomic DNA of *Chlorobium* BS-1 in these fluff samples was detectable but reached only $5.09 \cdot 10^{-3}$ and $2.75 \cdot 10^{-2}$ ng BS-1 DNA·(g sediment dry weight)$^{-1}$, respectively. No correlation existed between the DNA amount present in the sediment and the integrated amount of BChl*e* found in the overlying chemocline during our research cruise in summer 2007 ($r^2 = 0.0315$; Fig. 6). The molecular remains of BS-1 which were consistently detected in sediments at the periphery of the Black Sea basin suggest that the lateral dispersal of BS-1 cells from the zone of photosynthetic growth in the Black Sea centre towards the periphery does not represent a singular or transient event but rather is a long-lasting phenomenon.

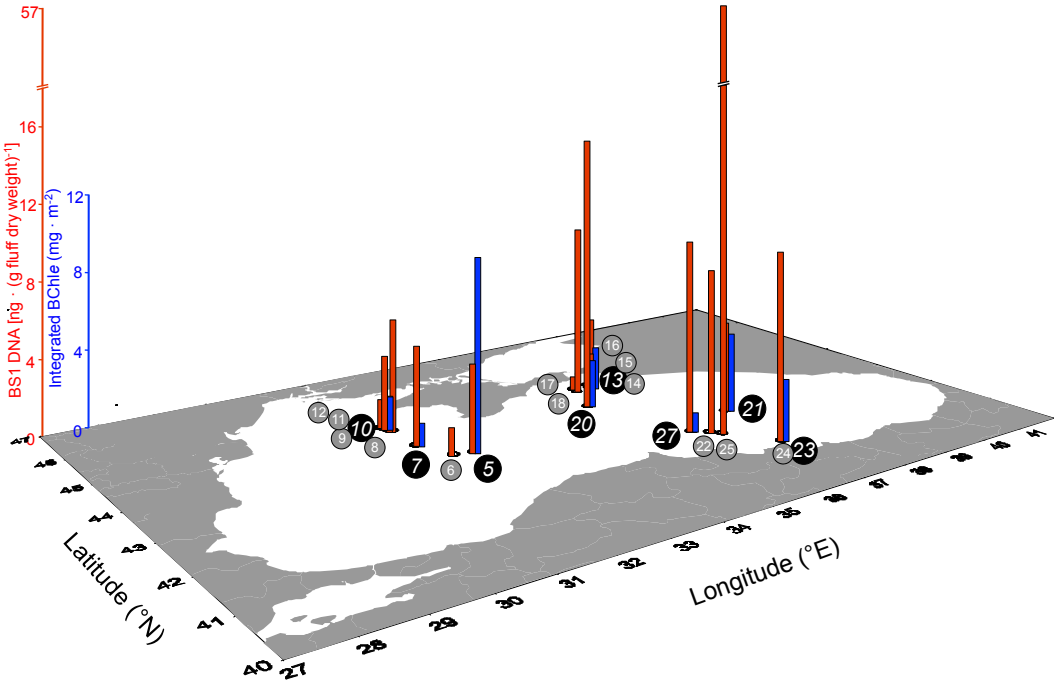

Figure 6. Concentration of genomic DNA of *Chlorobium* BS-1 determined in the fluff layer at the sediment surface across the Black Sea basin (red bars). For comparison, the integrated biomass (expressed as mgBChl*e*·m^{-2}, blue bars), where determined are given. Numbers of the sampling locations are provided. Main sampling stations are denoted by black points.

5.4.4 Survival of *Chlorobium* sp. BS-1

Based on physiological experiments with *Chlorobium* BS-1, the theoretical doubling time of the BS-1 cells would amount to 26 years during wintertime (Manske *et al.*, 2005). In addition, cultivation trials with the natural chemocline population of *Chlorobium* BS-1 have either failed (Hashwa & Trüper 1978, Gorlenko *et al.*, 2005) or indicated a very low fraction of culturable cells (Overmann *et al.*, 1992, Manske *et al.*, 2005). So far, it has remained unclear whether the population of *Chlorobium* BS-1 present in the periphery of the Black Sea or the cells persisting during wintertime consist of moribund cells or whether the cells are capable of maintaining their cell integrity and energy charge under such prolonged starvation. Therefore the survival and maintenance of *Chlorobium* BS-1 under starvation conditions was assessed.

When exponentially grown laboratory cultures of *Chlorobium* BS-1 were subjected to a prolonged incubation under anoxic conditions and in the absence of light and sulfide, the number of culturable cells showed a rapid decrease to 3 % of the initial value over the first 16 hours of incubation. Subsequently, the culturability decreased more slowly to 0.015% after 2 weeks. Culturability of cells exposed to an air atmosphere decreased more rapidly over the

first week but reached the same value after 14 days (Suppl. Fig. 3). An extended incubation for 16 weeks decreased the fraction of culturable cells only slightly further to 0.005% (data not shown). In contrast, a simultaneous exposure to oxygen and sulfide resulted in a rapid decrease in culturability over the first hour (Suppl. Fig. 3, rectangles). Total cell numbers of *Chlorobium* BS-1 remained constant throughout these experiments. Whereas the low culturability of starved laboratory cultures of *Chlorobium* BS-1 thus matches the very low cultivation success determined in chemocline samples (Overmann *et al.*, 1992, Manske *et al.*, 2005), non-culturable cells were still capable of maintaining cell integrity over 16 weeks which is significantly longer than for many other bacteria (e.g., Binnerup & Sørensen 1993, Cho & Kim 1999).

In order to study the capability of *Chlorobium* BS-1 cells of maintaining an energised state during this time period, the cellular ATP content in stationary phase cells was followed over 52 days (Fig. 7). Cells grown for 3 to 4 weeks under light saturation or light limitation were tested and compared to *Chlorobium phaeovibrioides* DSMZ 269^T. The latter represents the most low-light adapted strain of green sulfur bacteria known besides *Chlorobium* BS-1 (Overmann *et al.*, 1992). After growth under light saturation, stationary phase cultures of *Chlorobium* BS-1 maintained a constant level of cellular ATP over 52 days, if exposed to light intensities of 0.1 or 0.01 µmol quanta·m^{-2}·s^{-1}. In contrast, incubation at 0.0014 µmol quanta·m^{-2}·s^{-1} or in darkness over 28 days resulted in a significant ($p < 0.01$) decrease of cellular ATP to 62 and 57 % of the initial value, respectively. After 52 days, the cellular ATP content dropped further to 50% and 52%, respectively (Fig. 7A). By comparison, cells pregrown under light limitation were capable of maintaining a constant ATP level during the first 28 days at all light intensities, even when incubated at 0.0014 µmol quanta·m^{-2}·s^{-1}. Cells incubated in the dark showed a significant ($p < 0.01$) decrease to 55% of the initial ATP-concentration. After 52 days of incubation at 0.0014 µmol Quanta m^{-2} s^{-1}, the ATP level dropped down to 74% of the initial value, which was still significantly higher than values of dark incubated cultures ($p < 0.01$) (Fig. 7B). The differential effect seen for the two types of cultures cannot be attributed to differences in intracellular storage compounds since cultures of BS-1 are devoid of such compounds in the stationary phase (Overmann *et al.*, 1992). Cell numbers of *Chlorobium* BS-1 remained constant throughout the experiments with the high [(2.71±0.28)·10^8 ml^{-1}] and low light adapted [(3.23±0.30)·10^8 ml^{-1}] cultures. In contrast to *Chlorobium* BS-1, all cultures of *Chl. phaeovibrioides* DSMZ 269^T had a decreased cellular ATP content after 28 as well as 52 days of incubation, irrespective of the light intensity used during starvation (data not shown). Our data show that low-light adapted cells of *Chlorobium*

BS-1 are capable of maintaining the energised state significantly longer at a light intensity of 0.0014 µmol Quanta m^{-2} s^{-1} which corresponds to light intensities encountered by the cells at the periphery of the Black Sea during summer (0.0020 - 0.0027 at S23 and S10, respectively) or at the centre of the western gyre in winter (≤ 0.0022 µmol quanta·m^{-2}·s^{-1}; Manske *et al.*, 2005). This difference in the response towards 0.0014 µmol Quanta m^{-2} s^{-1} of low versus high light adapted BS-1 at least in part can be explained by the significantly higher specific BChl*e* content (Overmann *et al.*, 1992) of low light adapted cultures.

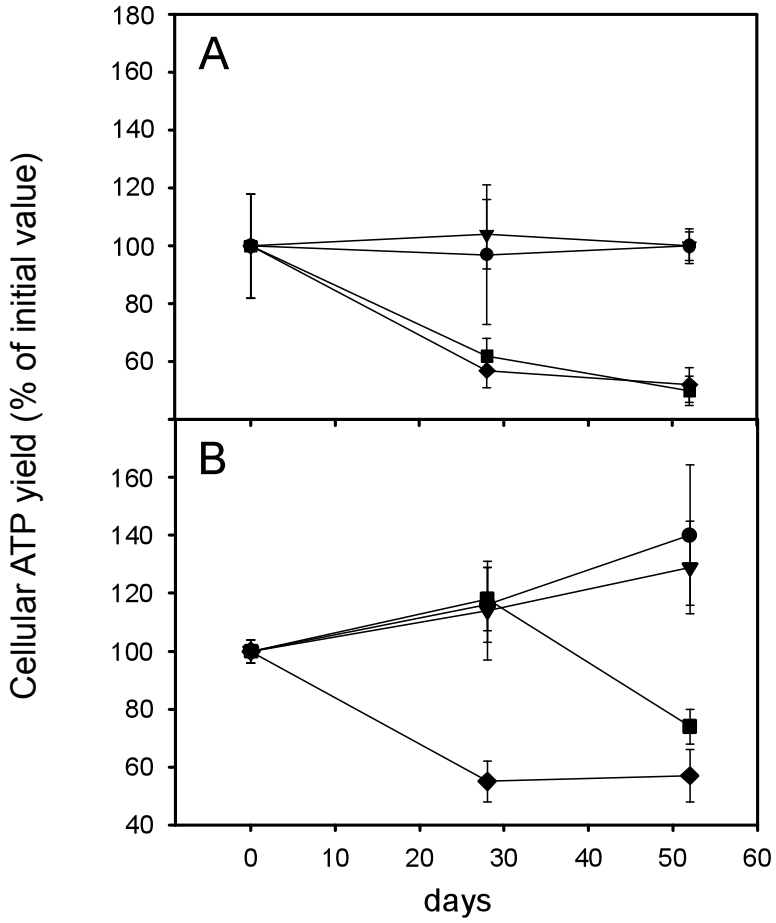

Figure 7. Time course of changes in ATP content of stationary *Chlorobium* BS-1 cultures. **A.** Cells precultivated at high (3 µmol quanta·m^{-2}·s^{-1}) light intensities. **B.** Cultures precultivated at low (0.1 µmol quanta·m^{-2}·s^{-1}) light intensities. Cells were incubated for 28 or 52 days at different light intensities of 0.1 (●), 0.01 (▼), 0.0014 (■) µmol quanta·m^{-2}·s^{-1} and in the dark (♦). Incubations at 10 and 1 µmol quanta·m^{-2}·s^{-1} during the starvation experiment yielded data very similar to those obtained at 1 and 0.1 µmol quanta·m^{-2}·s^{-1} (not shown). Vertical bars give 1 standard deviation.

However, first evidence for a more specific mechanism which is involved in the maintenance of *Chlorobium* BS-1 under extreme energy starvation can be derived from our pigment analyses of the natural samples. Green sulfur bacterial cells from deeper locations of the chemocline contained Bchl*e* homologs with longer aliphatic side chains (compare Suppl.Fig. 1). This shift has previously been documented for laboratory cultures of *Chlorobium* BS-1 after transfer to lower light intensities (Manske *et al.*, 2005). Similar to phylotype BS-1, a loss of [E,M]-Bchle_F with a concomitant increase in [I,E]-Bchle_F has been documented for *Chlorobium phaeobacteroides* strain Dagow III upon transfer to light limiting conditions (Glaeser *et al.*, 2002). Since the alkyl side chain of porphyrin ring III is directly involved in the aggregation of BChl molecules (van Rossum *et al.*, 2001), a high degree of alkylation leads to a red shift of the Q_Y absorption maximum by 7 to 11 nm (Borrego & Garcia-Gil, 1995). The red shift has been hypothesised to facilitate the channeling of excitation energy and hence increase the energy transfer efficiency from the chlorosomes towards the reaction centre (Borrego & Garcia-Gil, 1995). The fact that BS-1 cells from greater depths exhibited the same shift in the composition of Bchl*e* homologs suggests that the natural population physiologically responds, and possibly adapts, to the extremely low light conditions.

5.5 Conclusions

Based on the combined evidence gathered in the present study, a major fraction of the *Chlorobium* BS-1 population in the Black Sea is photosynthetically inactive, such that maintenance becomes a major determinant of the large scale distribution of the BS-1 population. The maintenance energy requirement of *Chlorobium* BS-1 can be estimated based on the minimum light intensity of 0.0014 μmol quanta·m^{-2}·s^{-1} which supports a stable cellular ATP content over 28 days and corresponds to $3.04 \cdot 10^{-7}$ kJ·m^{-2}·s^{-1} for daylight fluorescent tubes. The average absorption cross section of a single cell is the product of the specific absorption coefficient *in vivo* (0.020 m^2·mgBChl*e*$^{-1}$ for daylight fluorescent tubes; Overmann *et al.*, 1992) and the Bchl*e* content of the cells ($3.1 \cdot 10^{-5}$ ngBChle·$cell^{-1}$; Manske *et al.*, 2005), and hence amounts to 0.62 μm^2·$cell^{-1}$ for *Chlorobium* BS-1. Accordingly, a single *Chlorobium* BS-1 cell absorbs the light energy of $1.63 \cdot 10^{-14}$ kJ·$cell^{-1}$·d^{-1} in order to maintain its cellular ATP content over 28 days. Considering the limited energy transfer within the photosynthetic apparatus (10% from BChl*e* to the FMO protein; Otte *et al.*, 1991), the estimated maintenance energy requirement likely is in the range of $1.6 \cdot 10^{-15}$ kJ·$cell^{-1}$·d^{-1}. At a more favourable energy transfer efficiency of 30%, the maintenance energy requirement would amount to $4.9 \cdot 10^{-15}$ kJ·$cell^{-1}$·d^{-1}. These values are almost four orders of magnitude lower than that determined for the anoxygenic phototrophic *Rhodobacter capsulatus* KB1 ($8.02 \cdot 10^{-12}$ kJ·$cell^{-1}$·d^{-1}; Göbel, 1978), over hundredfold lower than values for several chemoorganoheterophic anaerobic bacteria (4.6 to $6.2 \cdot 10^{-13}$ kJ·$cell^{-1}$·d^{-1}; Adams *et al.*, 2006; Scholten & Conrad, 2000) and still tenfold lower than the lowest maintenance energy requirement reported so far (*Syntrophobacter fumaroxidans*; $2.8 \cdot 10^{-14}$ kJ·$cell^{-1}$·d^{-1}; Scholten & Conrad, 2000). The estimate determined for *Chlorobium* BS-1 thus represents the lowest value determined for laboratory bacterial cultures and indicates that BS-1 is particularly well adapted to long-term survival under the extreme low light conditions in the Black Sea chemocline. Considering the low metabolic activity of natural communities of bacteria in marine sediments (D´Hondt *et al.*, 2002), BS-1 may also serve as a suitable laboratory model for future studies of the cellular mechanisms of low maintenance energy requirements.

Lastly, its outstanding and specific adaptation to low light environments and its so far unique ITS sequence render *Chlorobium* BS-1 a suitable indicator organism for future paleoceanography studies. Since all described green sulfur bacteria are sulfide-dependent obligate photolithoautotrophs, fossil remains of their specific carotenoids isorenieratene and chlorobactene have often been employed as indicators of past photic zone anoxia in the marine environment (Passier *et al.*, 1999; Menzel *et al.*, 2002). Using this approach, subfossil

isorenieratene has been found in up to 6,000 year-old sediment layers of the Black Sea (Sinninghe Damsté *et al.*, 1993). However, the low diversity of carotenoids which occur in the over 100 different phylotypes of green sulfur bacteria does not allow to differentiate between bacteria occupying different ecological niches and hence preclude a more detailed reconstruction of past environmental conditions. In this respect, the highly sensitive ITS-specific qPCR approach established in the present study is well suited for the targeted detection of subfossil molecular remains of *Chlorobium* BS-1 as a specific indicator of deep photic zone anoxia. A rather limited low-light adaptation has been shown to occur by spontaneous frameshift mutation in the *bchU* gene of laboratory cultures of *Chlorobaculum parvum* NCIB 8327 (Broch-Due & Ormerod, 1978; Saga *et al.*, 2003, Maresca *et al.*, 2004). A similar change is observed in laboratory cultures of *Chlorobaculum tepidum* ATCC 49652^{T} after genetic inactivation of methyltransferase BchQ, BchR or BchU, resulting in the loss of a specific methyl-substituents in the bacteriochlorophyll molecule (Maresca *et al.*, 2004; Chew *et al.*, 2007). In contrast to these laboratory mutants, BS-1 differs fundamentally from other green sulfur bacteria by its larger chlorosomes (Fuhrmann *et al.*, 1993), much lower light saturated growth rates (Overmann *et al.*, 1992) and extraordinary low maintenance requirements (this study). While the genetic basis of these traits is currently not known, it is likely to be complex and hence not expected to change as rapidly as the methylation pattern of bacteriochlorophyll pigments. Provided that the extraordinary low-light adaptation of *Chlorobium* BS-1 has been a genetically stable trait over the past 6,000 years, the molecular approach developed in the present study will therefore aid in the reconstruction of the paleoceanography of the Black Sea or other anoxic marine basins.

5.6 Experimental procedures

5.6.1 Sampling

Sampling and *in situ* measurements were conducted during FS Meteor cruise M72 leg 5 from Istanbul to Istanbul between May 14 and June 5, 2007 covering 21 stations (Suppl. Table 1, Fig. 1A). Vertical profiles of environmental parameters and green sulfur bacterial biomass through the chemocline were determined at 8 main stations including 3 locations in the western basin and 5 sites in the less well studied eastern basin between the entry to the Sea of Azov (station 13) and the Kizirlimak estuary (station 23). At the main stations, water samples were collected with a SBE911 plus CTD system (Sea-Bird Electronics Inc., Washington, USA) and a SBE32 rosette equipped with 13 ten-liter FreeFlow-bottles (Hydrobios). At the other stations, a pump-CTD connected to a Hydrobios rosette with 12 five-liter FreeFlow-bottles (Hydrobios) was used. Samples for the determination of sulfide and BChl*e* concentrations were always taken from the same cast and the same sampling bottles. Sulfide concentrations were measured immediately after the cast on board (see below).

Surface sediments were obtained using a multicorer with 10 cm inner diameter plexiglass tubes. Immediately after sampling, the flocculent sediment surface layer (fluff) was collected with sterile syringes, transferred to a sterile 50 ml disposable screw cap tube, frozen and stored at -20°C.

5.6.2 Chemical and physical parameters

Conductivity, temperature and molecular oxygen concentrations were monitored with a SBE3plus temperature electrode, a SBE4 conductivity electrode and a SBE43 oxygen electrode (Sea-Bird Electronics) mounted to the rosette water sampler.

High sensitivity measurements of downwelling irradiance were conducted with a LI-190SZ quantum sensor connected to a LI-1400 data logger (LiCor Biosciences GmbH, Bad Homburg, Germany). Both were encased in a custom-built stainless steel pressure proof chamber which was sealed with a Perspex lid on top (Manske *et al.*, 2005). All measurements were performed within one hour around solar noon operating the data logger in the integration mode. The instrument was lowered at 5 m-intervals through the chemocline and the quantum flux recorded in 5 replicate measurements by integrating the light quanta over one minute time intervals. By this method, the detection limit was lowered to $(9.9 \pm 2.6) \cdot 10^{-4}$ µmol quanta$\cdot m^{-2} \cdot s^{-1}$. In order to correct for short term changes in incident light during the *in situ* measurements, global irradiance was recorded in parallel by the ship pyranometer and the quantum sensor calibrated against the ship pyranometer. The vertical attenuation

coefficient k_D was calculated from the slope in plots of the natural logarithm of underwater light quantum flux *versus* depth.

Sulfide concentrations were determined by a modification of the method of Cline (1969). In order to prevent chemical oxidation with atmospheric oxygen, water samples were transferred from the FreeFlow bottles directly into 25 ml volumetric flasks containing 5 ml of fixative (1% w/v ZnAc and 0.5 M NaOH) using gas-tight tubing and by inserting the end of the tube directly into the fixative. After addition of 2.5 ml 0.2% (w/v) N,N-dimethyl-*p*-phenylene diammonium dichloride and 0.125 ml of a 10% (w/v) Fe(III) ammonium sulfate solution, the samples were incubated for 20 min in the dark, the volume adjusted to 25 ml with double distilled H_2O, and the absorbance of the sample was read at 670 nm. The detection limit of this method was 0.2 µM sulfide.

Dry weights of the sediment fluff samples were determined after drying over night at 130°C.

5.6.3 Pigment analysis

Three liters of water from each depth were filtered onto 0.22 µm pore size Isopore polycarbonate filters (Millipore, Eschborn, Germany; diameter 47mm). Filtration was conducted in the dark. Filters were immediately frozen and subsequently stored at -20°C. Prior to pigment extraction and quantification, the required glass equipment was rinsed with a mixture of MeOH/acetone (2:7). The filters were cut into pieces and transferred into 10 ml Pyrex vials. After addition of 3.5 ml MeOH/acetone (2:7, v/v), each vial was sonicated for 15 min in a ultrasonic bath (Bandelin Sonorex, Bandelin Electronic, Berlin, Germany) and pigments were extracted overnight at 4 °C. The extracts were filtered through a 0.22 µm filter (Multoclear-13 PTFE, CS-Chromatographie Service GmbH, Langerwehe, Germany) and then evaporated under a stream of N_2. Dried pigments were stored in brown glass tubes at -20°C until further analysis. Bacteriochlorophylls were quantified by HPLC as described earlier (Manske *et al.*, 2005). For standardisation, BChl*e* was extracted from *Chlorobium* BS-1 and the concentration of BChl*e* in the extract was determined photometrically at 649 nm employing the molar extinction coefficient of 48.9 $mM^{-1}cm^{-1}$ (Borrego *et al.*, 1999). BChl*e* homologs were identified based on their retention time and absorption spectra (Glaeser *et al.*, 2002; Repeta & Simpson, 1991). The total amount of BChl*e* was calculated from the sum of all detected homologs.

5.6.4 Extraction of DNA and RNA

At stations 5, 10 and 21, microbial cells from above, within and below the chemocline were enriched by means of *in situ* pumps (McLane, Falmouth, USA) which were loaded with 142 mm diameter GF/F glass fibre filters (Whatman GmbH, Dassel, Germany) and deployed at different water depths (as given in the Results section) for 3 hours to collect between 300 and 700 litres of water. After retrieval of the pumps, glass fibre filters were placed on a cooled metal plate and 17 mm diameter filter discs were punched out, cut into in 4 mm wide strips and frozen.

Genomic DNA was extracted by a modified version of the method of Fuhrman *et al.* (1988). The filter strips were first boiled in 3 ml STE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA, 100 mM NaCl) with 1% SDS and then in 1.5 ml TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA) buffer for 10 min. The extracts were combined and mixed with 4 ml phenol:chloroform:isoamylalcohol (25:24:1; v/v/v), incubated for 15 min at room temperature and centrifuged for 10 min at 5,500 x g. The upper phase was reextracted with 4 ml chloroform and purified by dialysis in 5 mM Tris·HCl in Centricon-50 units (Millipore, Eschborn, Germany). Genomic DNA was precipitated with 100% ethanol and 0.3 M sodium acetate at -20 °C overnight and finally washed with 70 % (v/v) ice-cold ethanol. DNA concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen, Karlsruhe, Germany).

RNA was recovered using a modified version of the method of Eicher *et al.* (2004). Filter strips were combined with 1 g of 0.1 mm diameter siliconised zirconia beads (BioSpec Products, Bartlesville, USA), 600 µl extraction buffer (50 mM sodium acetate, 10 mM EDTA, 1 % SDS, pH 4.2) and 600 µl acidic phenol and the cells were disrupted in a beadbeater (BioSpec Products, Bartlesville, USA) for 5 min. The homogenate was centrifuged for 30 min at 16,000 x g and 4°C, and subsequently extracted twice with the same volume of chloroform and 0.3 M sodium acetate. The RNA was precipitated overnight at -20 °C, centrifuged for 30 min at 18,000 x g and 4°C, and washed with 70 % ethanol. After degradation of the DNA with DNase I (Fermentas, St. Leon-Roth, Germany) the RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The RNA concentration was assessed with the nanodrop ND-1000 (peglab, Erlangen, Germany). RNA quality was checked on 1.2 % agarose gels containing 1.1 % formaldehyde (Kroczek & Siebert, 1990). All solutions were prepared with deionised water and treated with 0.1 % diethyl pyrocarbonate overnight before autoclaving.

Genomic DNA from sediment samples was extracted using the Ultra Clean Soil™ DNA Kit (Mobio Laboratories, Inc., Solana Beach, CA) according to the instructions of the manufacturer, using 0.4 ml aliquots of each fluff sample.

5.6.5 qPCR of *rrn* operons and RT-qPCR of *rrn* transcripts

The genome of *Chlorobium* BS-1 harbours two *rrn* operons (http://genome.jgi-psf.org/chlpb/chlpb.home.html). The ITS regions of both operons are 504 bp long and identical in sequence. A highly specific primer set consisting of BS1-ITS38f (5´-GCTCAAAGAGTAATGGTCC-3´) and BS1-ITS518r (5´-GCATTTCATTCTTACCAGCT-3´) was developed for the quantification of BS-1 genomes, or of cDNA of ITS-transcripts by qPCR. This primer set targets the ITS region exclusively of *Chlorobium* BS-1 and was employed at a concentration of 200 nM each. To each reaction, bovine serum albumine was added at a concentration of 0.4 $\mu g \cdot \mu l^{-1}$. PCR was performed in 25 µl reactions using the iQ SYBR Green Supermix (Biorad) and a Biorad iCycler. Each reaction contained between 0.5 and 5 ng of DNA extract or the cDNA equivalent to 9 ng RNA. PCR conditions were 95°C for 5 min, 45 cycles consisting of 94°C for 30s, melting at 63°C for 45 s, and extension at 72°C for 1 min, and a final step of 10 min at 72°C.

In the sediment samples, the copy numbers of ITS-DNA were determined using 20 ng of DNA per assay. For water column samples, the copy numbers of the BS-1 ITS region was determined for DNA extracts as well as for cDNA. cDNA was produced from 60 ng RNA by reverse transcription with the ImProm-II™ Reverse Transcriptase (Promega, Mannheim, Germany) and random hexamer primers according to the instructions of the manufacturer. For standardisation, the copy number of eubacterial 16S rRNA genes was quantified in parallel for the water column samples, using the eubacterial primer set 341-mod-f (5´-CCTACGGGWGGCWGCAG-3´; modified after Muyzer *et al.*, 1998) and uni-515-r (5´-CCGCGGCTGCTGGCAC-3´; modified after Lane 1991) at a concentration of 160 nM each. To each reaction, bovine serum albumine was added at a final concentration of 0.4 $\mu g \cdot \mu l^{-1}$. PCR conditions were 95°C for 3 min, followed by 40 cycles consisting of 94°C for 30s, melting at 64°C for 20 s, and extension at 72°C for 45 sec. For calibration, a plasmid harbouring the cloned 16S rRNA gene and the ITS1 region of BS-1 was employed. Each quantification was performed in three parallels.

5.6.6 Photosynthetic activity of the natural BS-1 population

Two stations in the centre of the western (station 5; 43°22'N 32°38,0'E) and the eastern (station 21; 42°45,0'N 37°30,0'E) basin were chosen for the determination of the photosynthesis-irradiance curve of the natural population. Since the chemocline is at its most shallow position at these central locations (Oguz *et al.*, 1994), the corresponding samples were expected to yield the highest photosynthetic rates. Highly concentrated biomass samples were obtained using the *in situ* pumps described above and the amount of BChl*e* per filter area determined as described above. After recovering the pumps, the filters were removed and directly transferred to an anaerobic glove box. Subsequently, 25 mm diameter filter discs were punched out using a cork borer and the discs were transferred, cell side to bottom, into 22 ml glass scintillation vials filled with chemocline water. Based on the BChl*e* concentration of the water samples (Suppl. Fig. 2) and the BChl*e* content of *Chlorobium* BS-1 cells in situ (Manske *et al.*, 2005), it was calculated that the BS-1 cells formed a monolayer on the filter discs. Therefore selfshading of the phototrophic cells is unlikely to occur in our experimental setup. The vials were then closed with teflon-coated butyl rubber septa and screw caps. To allow a sufficient supply of the bacterial cells with CO_2 and H_2S during the incubation experiment, sterile glass spacers were placed underneath the filter discs at the bottom of each vial.

The incorporation experiment was started by spiking each vial with 370 kBq of $NaH^{14}CO_3$ (Hartmann Analytics, Braunschweig, Germany). After mixing, the vials were incubated for 17 h in a light cabinet consisting of 6 chambers providing light intensities between 18.1 and 0.0033 µmol quanta$\cdot m^{-2}\cdot s^{-1}$ (Manske *et al.*, 2005). The light cabinet included a dark chamber for the incubation of the controls for nonphotosynthetic incorporation of CO_2 and kept at a constant value of 15 °C corresponding to the in situ temperature. The compartments were illuminated from below by two daylight fluorescent tubes (Osram daylight 5000 de luxe, 18W). At each light intensity, 3 parallels were incubated. The three dark controls were wrapped in aluminium foil and put into the dark chamber. As a control for adsorption and other abiotic processes, parallel samples were fixed with 2.5% (w/v) paraformaldehyde in chemocline water prior to incubation. After incubation, the filters were recovered, dried gently by suction on a filter holder and washed twice with sterile water. The washed filters were transferred to fresh scintillation tubes and inorganic carbon was removed from the filters by acidification with 5 drops of 1M HCl and further incubation of the closed vials for 3 h. The filters were subsequently air dried and 10 ml Ultima Gold F scintillation liquid for filters (Packard Bioscience, Groningen, NL) were

added for liquid scintillation counting. Total radioactivity in each incubation vial was determined by mixing a 0.5 ml aliquot with 10 ml scintillation liquid (Ultima Gold; Packard). Radioactivity was determined in a Tricarb 2199 TR scintillation counter (Canberra Packard GmbH, Dreieich, Germany).

5.6.7 Survival and maintenance of the cellular ATP content in the dark

For oxygen exposure experiments, exponentially growing cultures of *Chlorobium* BS-1 were harvested by centrifugation and the cells were resuspended in artificial sea water medium (Coolen & Overmann, 2000) which had been adjusted to the ionic strength of the Black Sea chemocline. All manipulations of cell suspensions were performed under a N_2 atmosphere in an anaerobic chamber and using anoxic media. The resuspended cells were diluted 1:100 in adjusted artificial sea water medium and the suspension transferred to a sterile serum flask (anoxic incubations, oxic incubations with sulfide) or Erlenmeyer flasks with cotton plugs (oxic incubations).

Incubation experiments were conducted at 15°C in the dark and lasted for up to 16 weeks. For anoxic incubation, the head space of the serum flasks was flushed with sterile N_2 and the flasks sealed gas tight. Additional parallels in serum flasks received neutralised sulfide solution at a final concentration of 2.5 mM after flushing the headspace with sterile air, to simulate radical formation by the simultaneous presence of sulfide and oxygen. For oxic cultures, 80 ml aliquots of the cell suspension were incubated in baffled 250 ml Erlenmeyer flasks closed with cotton plugs and incubated on a rotary shaker operated at 250 rpm. At different time points, subsamples for the determination of total cell numbers and the number of viable cells were taken. For the determination of the total cell numbers, cells were fixed with 2% (v/v) glutardialdehyde, the fixed cells were stained with 4',6-diamidino-2-phenylindol (DAPI) and counted on black polycarbonate filters (pore size 0.22 µm; Millipore) by epifluorescence microscopy. The number of viable cells was determined in most probable number series (Beatty *et al.*, 2005), using artificial sea water medium supplemented with 2.5 mM sulfide and 1 mM malate. For comparison, a culture of *Chlorobium* strain GSB1 (Beatty *et al.*, 2005) was used.

Intracellular ATP concentrations were determined in stationary phase cultures of BS-1 after different time intervals of incubation. Cultures were pregrown either under a light intensity of 3 µmol Quanta$\cdot m^{-2}\cdot s^{-1}$ (light saturation of growth) or 0.1 µmol Quanta$\cdot m^{-2}\cdot s^{-1}$ (light limitation of growth). For comparison, *Chlorobium phaeovibrioides* DSM 269^T was used. Cultures were incubated in two parallels at each light intensity. The cellular ATP-

content was determined after 28 and 52 days of incubation in the stationary phase and the cell numbers were counted with a Neubauer chamber (Paul Marienfeld GmbH, Lauda-Königshofen, Germany).

Cellular ATP content was determined in 1 ml culture aliquots. The samples were first boiled for 8 min in 9 ml of 20 mM Tris·HCl (pH 7.7) (Holm-Hansen & Booth, 1966). 100 µl of the sample were then combined with 900 µl buffer (40 mM Tris·HCl, pH 8.2; 10 mM KH_2AsO_4, 1 $mg \cdot ml^{-1}$ BSA, 10 mM Mg acetate, 2 µM pyrophosphate and 0.1 mM dithiotreithol), 16 µg luciferin and 2 µg luciferase (P.J.K. GmbH, Kleinblittersdorf, Germany). Luminescence was read with a Luminometer (Berthold, Bad Wildbad, Germany) over a 20 s measuring interval and quantified employing a standard curve. Blank values were determined before each measurement and internal standards (adding a defined amount of ATP to the samples) were used in order to control for quenching.

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5.8 References

Adams, C.J., Redmond, M.C. & Valentine, D.L. (2006). Pure-culture growth of fermentative bacteria, facilitated by H_2 removal: bioenergetics and H_2 production. *Appl Environ Microbiol* **72,** 1079-1085

Beatty, J.T., Overmann J., Lince, M.T., Manske, A.K., Lang, A.S., Blankenship, R.E., VanDover, C.L., Martinson, T.A. & **Plumley, G.F. (2005).** An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. *Proc Natl Acad Sci* **102,** 9306-9310jj

Bergstein, T., Henis, Y. & Cavari, B.Z. (1979). Investigations on the photosynthetic sulfur bacterium *Chlorobium phaeobacteroides* causing seasonal blooms in Lake Kinneret. *Can J Microbiol* **25,** 999-1007

Binnerup, S.J. & Sørensen, J. (1993). Long-term deficiency in *Pseudomonas aeruginosa* PAO303 results in cells which are non-culturable under aerobic conditions. *FEMS Microbiol Ecol* **13,** 79-84

Borrego, C. M. & Garcia-Gil, L. J. (1995). Rearrangement of light harvesting bacteriochlorophyll homologs as a response of green sulfur bacteria to low light intensities. *Photosynth Res* **41,** 53-65.

Borrego, C. M., Arellano, J. B., Abella, C. A., Gillbro, T. & Garcia-Gil, L. J. (1999). The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroide*s. *Photosynth Res* **60,** 257-264

Broch-Due, M. & Ormerod, J.G. (1978). Isolation of a BChl*c* mutant from *Chlorobium* with BChl*d* by cultivation at low light intensity. *FEMS Microbiol Lett* **3:** 305-308

Chew, A. G. M., Frigaard, N.-U. & Bryant, D.A. (2007). Bacteriochlorophyllide *c* C-8^2 and C-12^1 methyltransferases are essential for adaptation to low light in *Chlorobaculum tepidum*. *J. Bacteriol.* **189,** 6176-6184

Cho, J.-C. & Kim, S.-J. (1999). Viable, but non-culturable, state of a green fluorescence protein-tagged environmental isolate of *Salmonella typhi* in groundwater and pond water. *FEMS Microbiol Lett* **170,** 257-264

Cline, J. D. (1969). Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol Oceanogr* **14:** 454-458.

Coble, P.G., Gagosian, R.B., Codispoti, L.A., Friederichs, G.E. & Christensen, J.P. (1991). Vertical distribution of dissolved and particulate fluorescence in the Black Sea. *Deep-Sea Res* **38,** 985-1001

Codispoti, L. A., Friederich, G. E., Murray, J. W. & Sakamoto, C. M. (1991). Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. *Deep-Sea Res* **38,** 691-710.

Coolen, M. J. L. & Overmann, J. (2000). Functional exoenzymes as indicators of metabolically active bacteria in 124.000-year-old sapropel layers of the eastern Mediterranean Sea. *Appl Environ Microbiol* **66,** 2589-2598.

Coolen, M. J. L. & Overmann, J. (2007). 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. *Environ Microbiol* **9,** 238-249.

D´Hondt, S., Rutherford, S. & Spivack, A.J. (2002). Metabolic activity of subsurface life in deep-sea sediments. *Science* **295,** 2067-2070

Eicher, S, Weinbauer, M.G., Dominik, K. & Höfle, M. (2004). Extraction of total RNA and DNA from bacterioplankton. In: Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D., van Elsas, J.D. (Eds.), *Molecular Microbial Ecology Manual*. p. 103-120.

Fuhrman, J. A., Comeau, D. E., Hagström, Å. & Chan, A. M. (1988). Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. *Appl Environ Microbiol* **54,** 1426-1429.

Fuhrmann S., Overmann J., Pfennig N. & Fischer U. (1993). Influence of vitamin B_{12} and light on the formation of chlorosomes in green- and brown-colored *Chlorobium* species. *Arch Microbiol* **160,** 193-198

Glaeser, J., Baneras, L., Rütters, H. & Overmann, J. (2002). Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. *Arch Microbiol* **177,** 475-485.

Glaeser, J., Sterz, M. & Overmann, J. (2003). Green sulfur bacteria in the chemocline. In: Hemleben,C., Hoernle, K., Jørgensen, B.B., Roether, W. (eds.) *METEOR-Berichte 03-1*, Ostatlantik-Mittelmeer-Schwarzes Meer, Cruise No. 51. Leitstelle METEOR Institut für Meereskunde der Universität Hamburg, p. 4-43 to 4-48.

Glazer, B. T., Luther III, G. W., Konovalov, S. K., Friederich, G. E., Trouwborst, R. E. & Romanov, A. S. (2006). Spatial and temporal variability of the Black Sea suboxic zone. *Deep-Sea Res II* **53,** 1756-1768.

Göbel, F. (1978). Quantum efficiencies of growth. In: R.K. Clayton, W.R. Sistrom (eds.) *The Photosynthetic Bacteria*. Plenum Press, New York, London. p. 907-934.

Gorlenko, V.M., Mikheev, P.V., Rusanov, I.I., Pimenov, N.V. & Ivanov, M.V. (2005). Ecophysiological properties of photosynthetic bacteria from the Black Sea chemocline zone. *Microbiology* **74,** 201-209 (Translated from Mikrobiologiya 74: 239-247).

Gulin, S.B. (2000). Recent changes of biogenic carbonate deposition in anoxic sediments of the Black sea: sedimentary record and climatic implication. *Mar Environ Res* **49,** 319-328.

Hashwa, F.A. & Trüper, H.G. (1978). Viable phototrophic sulfur bacteria from the Black-Sea bottom. *Helgoländer wiss. Meeresunters* **31,** 249-253.

Holm-Hansen, O. & Booth, C. R. (1966). The measurement of adenosine triphosphate in the ocean and its ecological significance. *Limnol Oceanogr* **11,** 510-519.

Jewson, D.H., Talling, J.F., Dring, M.J., Tilzer, M.M., Heaney, S.I. & Cunningham, C. (1984). Measurements of photosynthetically available radiation in freshwater: comparative tests of some current instruments used in studies of primary production. *J Plankton Res* **6,** 259-273.

Jørgensen, B.B., Fossing, H., Wirsen, C.O. & Jannasch, H.W. (1991). Sulphide oxidation in the anoxic Black Sea chemocline. *Deep-Sea Res* **38,** 1081-1103.

Koblizek, M., Falkowski, P.G. & Kolber, Z.S. (2006). Diversity and distribution of photosynthetic bacteria in the Black Sea. *Deep-Sea Res II* **53,** 1934-1944.

Kroczek, R.A. & Siebert, E. (1990). Optimization of Northern analysis by vacuum-blotting, RNA-transfer visualization, and ultraviolet fixation. *Anal Biochem* **184,** 90-95.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In: *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E., Goodfellow, M (eds.). John Wiley, Chichester. p. 115-175.

Lin, X., Wakeham, S.G., Putnam, I.F., Astor, Y.M., Scranton, M.I., Chistoserdow, A.Y. & Taylor, G.T. (2006). Comparison of vertical distributions of prokaryotic assemblages in the anoxic Cariaco Basin and Black Sea by use of fluorescence in situ hybridization. *Appl Environ Microbiol* **72,** 2679-2690.

Manske, A. K., Glaeser, J., Kuypers, M. M. M. & Overmann, J. (2005). Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. *Appl Environ Microbiol* **71,** 8049-8060.

Manske, A. K., Henßge, U., Glaeser, J. & Overmann, J. (2008). Subfossil 16S rRNA gene sequences of green sulfur bacteria in the Black Sea and their implications for past photic zone anoxia. *Appl Environ Microbiol* **74,** 625-632.

Maresca, J.A., Chew, A.G.M., Ponsati, M.R., Frigaard, N.-U., Ormerod, J.G. & Bryant, D.A. (2004). The *bchU* gene of *Chlorobium tepidum* encodes the C-20 methyltransferase in bacteriochlorophyll *c* biosynthesis. *J Bacteriol* **186,** 2258-2566 .

Menzel, D., Hopmans, E. C., van Bergen, P. F., de Leeuw, J. W. & Sinninghe Damsté, J. S. (2002). Development of photic zone euxinia in the eastern Mediterranean Basin during deposition of Pliocene sapropels. *Mar Geol* **189,** 215-226.

Montesinos, E., Guerrero, R., Abella, C. & Esteve, I. (1983). Ecology and physiology of the competition for light between *Chlorobium limicola* and *Chlorobium phaeobacteroides* in natural habitats. *Appl Environ Microbiol* **46,** 1007-1016.

Murray, J. W., Jannasch, H. W., Honjo, S., Anderson, R. F., Reeburgh, W. S., Top, Z., Friederich, G. E., Codispoti, L. A. & Izdar, E. (1989). Unexpected changes in the oxic/anoxic interface in the Black Sea. *Nature* **338,** 411-413.

Murray, J.W., Codispoti, L.A., Friederich, G.E. (1995). Oxidation-reduction environments: the suboxic zone in the Black Sea. *In*: Huang, C.P., O´Melia, C.R. & Morgan, J.J. (eds). *Aquatic Chemistry: Interfacial and interspecies processes*, vol. 244. American Chemical Society, Washington, DC, pp. 157-176.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H. & Wawer, C. (1998). Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. *In* Akkermans, A.D.L., van Elsas, J.D., and de Bruijn, F.J. (eds.), *Molecular microbial ecology manual*, vol. 3.4.4. Kluwer Academic Publishers, Dordrecht, The Netherlands. p. 1 - 27

Neretin, L. N., Abed, R. M. M., Schippers, A., Schubert, C. J., Kohls, K. & Kuypers, M. M. M. (2007). Inorganic carbon fixation by sulfate-reducing bacteria in the Black Sea water column. *Environ Microbiol* **9,** 3019-3024.

Oguz, T. & Besiktepe, S. (1999). Observations on the rim current structure, CIW formation and transport in the western Black Sea. *Deep-Sea Res I* **46,** 1733-1753.

Oguz, T., La Violette, P. E. & Unluata, U. (1992). The Upper Layer Circulation of the Black Sea: Its Variability as Inferred From Hydrographic and Satellite Observations. *J Geophys Res* **97** (C8), 12.569-12.584.

Oguz, T., Latun, V. S., Latif, M. A., Vladimirov, V. V., Sur, H. I., Markov, A. A., Pzsoy, E., Korovshchikov, B. B., Eremeev V. V. & Ünlüata, Ü. (1993). Circulation in the surface and intermediate layers of the Black Sea. *Deep-Sea Res I* **40,** 1597-1612.

Oguz, T., Aubrey, D. G., Latun, V. S., Demirov, E., Koveshnikov, L., Sur, H. I., Diaconu, V., Besiktepe, S., Duman, M., Limeburner, R. & Eremeev, V. (1994). Mesoscale circulation and thermohaline structure of the Black Sea observed during HydroBlack ´91. *Deep-Sea Res I* **41.** 603-628.

Otte, S. C. M., van der Heiden, J. C., Pfennig, N. & Amesz, J. (1991). A comparative study of the optical characteristics of intact cells of photosynthetic green sulfur bacteria containing bacteriochlorophyll *c*, *d* or *e*. *Photosynth Res* **28,** 77-87.

Overmann, J. (2001). Green sulfur bacteria. In *Bergey´s Manual of Systematic Bacteriology*, 2nd edn, Vol. 1. Boone, D. R., and Castenholz, R. W. (eds). Baltimore, MD, USA: Williams & Wilkins, pp. 601-630.

Overmann, J., Cypionka, H. & Pfennig, N. (1992). An extremely low-light-adapted green sulfur bacterium from the Black Sea. *Limnol Oceanogr* **37,** 150-155.

Overmann, J. & Manske, A.K. (2006). Anoxygenic phototrophic bacteria in the Black Sea chemocline. In: Neretin, L.N. (ed.) *Past and present water column anoxia*. NATO ASI series. Springer-Verlag, Berlin. pp. 543-561.

Passier, H. F., Bosch, H. J., Nijenhuis, I. A., Lourens, L. J., Böttcher, M. E. & Leenders, A. (1999). Sulfidic Mediterranean surface water during Pliocene sapropel formation. *Nature* **397,** 146-149.

Pfennig, N., & Trüper, H. G. (1989). Anoxygenic phototrophic bacteria. In *Bergey´s Manual of Systematic Bacteriology*, Vol. III. Stanley, J. T., Bryant, M. P., Pfennig, N., and Holt, J. G. (eds). Baltimore, MD, USA: Williams & Wilkins, pp. 1635-1709.

Pimenov, N.V., Rusanov, I.I., Yusupov, S.K., Fridrich, J., Lein, A.Yu., Wehrli, B. & Ivanov, M.V. (2000). Microbial processes at the aerobic-anaerobic interface in the deep-water zone of the Black Sea. M*icrobiology* **69,** 436-448 (Translated from Mikrobiologiya 69: 527-540).

Repeta, D. J., Simpson, D. J., Jørgensen, B. B. & Jannasch, H. W. (1989). Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. *Nature* **342,** 69-72.

Repeta, D. J. & Simpson, D. H. (1991). The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. *Deep-Sea Res* **38,** 969-984.

Saga, Y., Oh-Oka, H. & Tamiaki, H. (2003). Presence of exclusively bacteriochlorophyll *c*-containing substrain in the culture of green sulfur photosynthetic bacterium *Chlorobium vibrioforme* NCIB 8327 producing bacteriochlorophyll *d*. *Anal Sci* **19,** 1575-1579.

Schmid, M., Schmitz-Esser, S., Jetten, M. & Wagner, M. (2001). 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and *in situ* detection. *Environ Microbiol* **3,** 450-459.

Scholten, J. C. M. & Conrad, R. (2000). Energetics of syntrophic propionate oxidation in defined batch and chemostat cocultures. *Appl Environ Microbiol* **66,** 2934-2942.

Sinninghe Damsté, J. S., Wakeham, S. G., Kohnen, M. E. L., Hayes, J. M. & de Leeuw, J. W. (1993). A 6,000 year sedimentary molecular record of chemocline excursions in the Black Sea. *Nature* **362,** 827-829.

Sorokin, Y. I. (2002). *The Black Sea. Ecology and oceanography*. Backhuys Publishers. Leiden. The Netherlands.

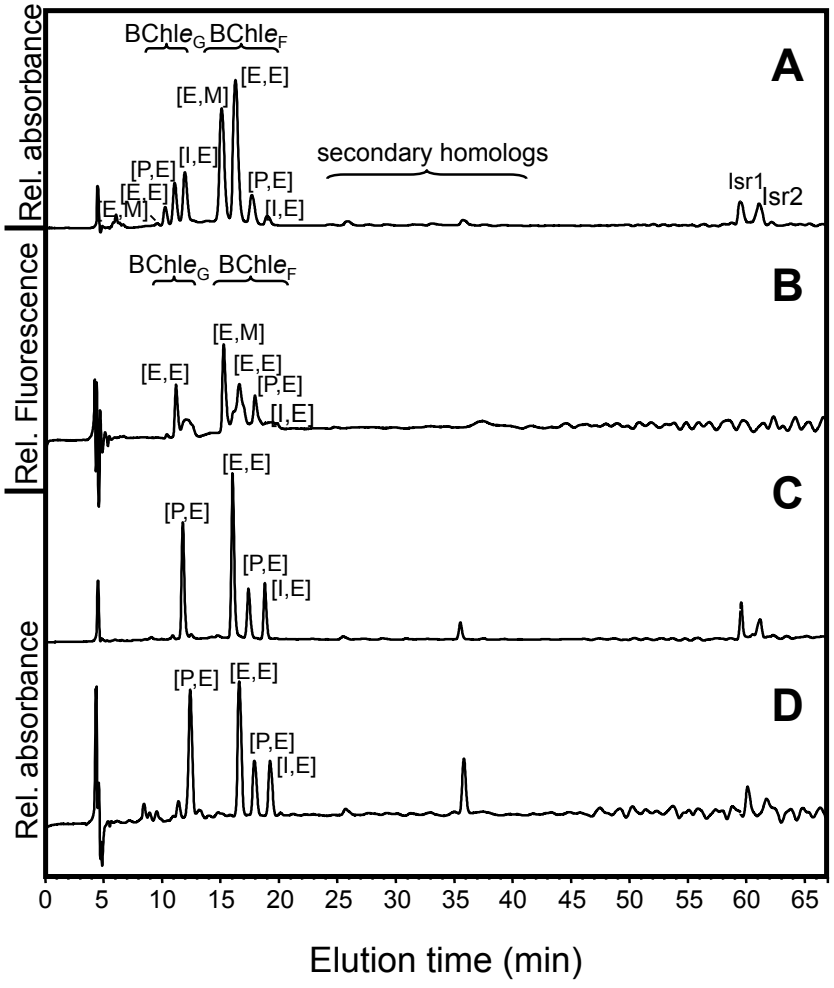
van Rossum B-J., D. B. Steensgard, F. M. Mulder, G. J. Boender, K. Schaffner, A. R. Holzwarth & de Groot, H. J. M. (2001). A refined model of the chlorosomal antennae of the green sulfur bacterium *Chlorobium tepidum* from proton chemical shift constraints obtained with high-field 2-D and 3-D MAS NMR dipolar correlation spectroscopy. *Biochemistry* **40,** 1587–1595.

Yakushev, E. V., Chasovnikov, V. K., Debolskaya, E. I., Egorov, A. V., Makkaveev, P. N., Pakhomova, S. V., Podymov,, O. I. & Yakubenko, V. G. (2006). The northeastern Black Sea redow zone: Hydrochemical structure and its temporal variability. *Deep-Sea Res II* **53,** 1769-1786.

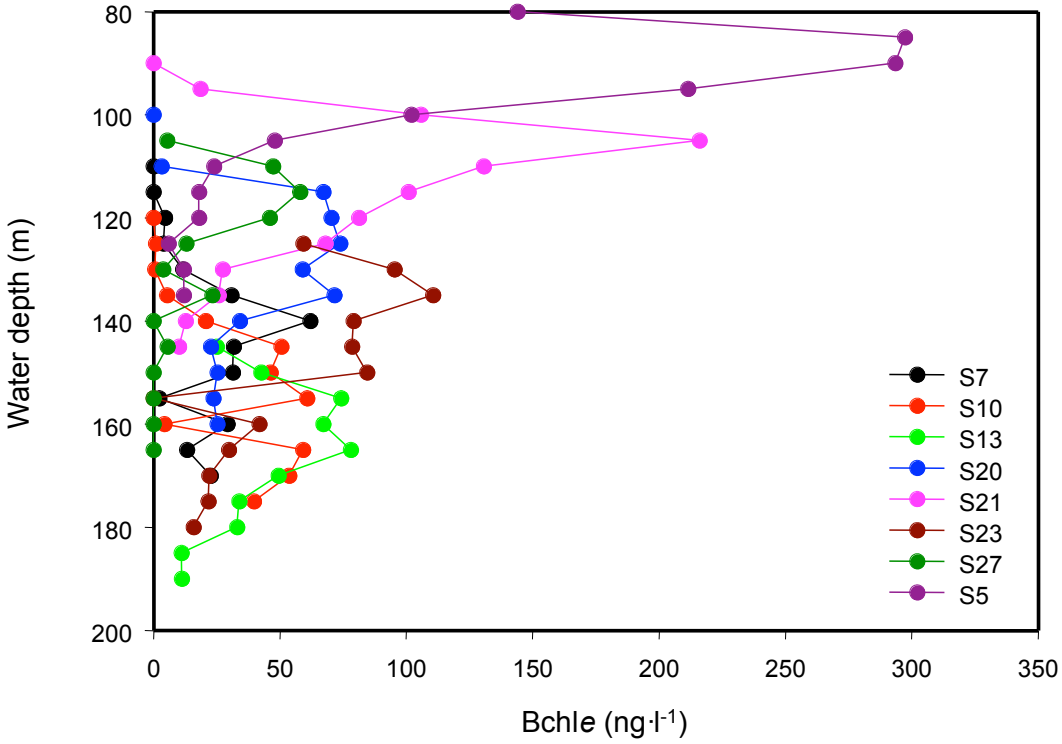
Yilmaz, A., Y. Çoban-Yildiz, F. Telli-Katakoç & Bologa, A. (2006). Surface and mid-water sources of organic carbon by photoautotrophic and chemoautotrophic production in the Black Sea. *Deep-Sea Res II* **53,** 1988-2004

5.9 Supplementary Figures

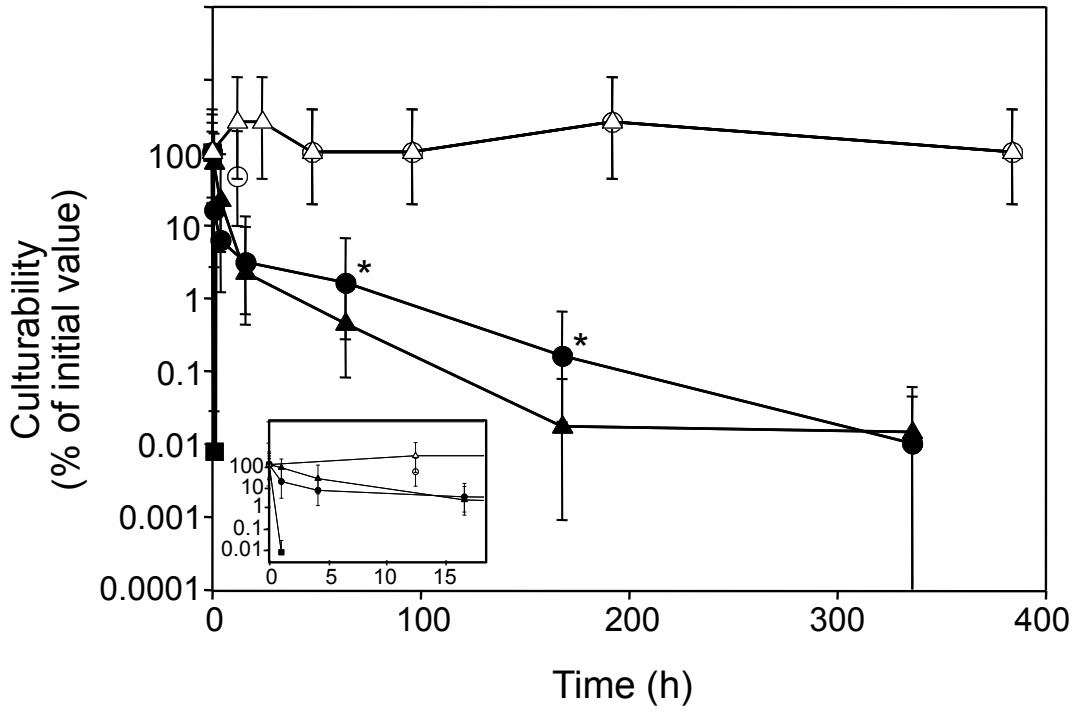
Supplementary Figure 1. Comparison of high-pressure liquid chromatography traces of photosynthetic pigments extracted from BS-1 enrichment cultures and from a chemocline water samples from different sampling locations. **A.** Pigment extract from a BS-1 culture grown at 3 µmol quanta·m^{-2}·s^{-1}. **B.** Pigment extract from particles collected in a chemocline sample from 85 m at station 5. Due to the lower amount of water sample available and the resulting low pigment concentrations, tetrapyrrol pigments could only be detected by fluorescence. Excitation wavelength was set to 476 nm, emission was recorded at 655 nm. **C.** Pigment extract from particles collected in a chemocline sample from 110 m at station 21. **D.** Pigment extract from particles collected in a chemocline sample from 135 m at station 23. The geraniol homologs ethyl/methyl [E,M]-BChl e_G, ethyl/ethyl [E,E]-BChl e_G, propyl/ethyl [P,E]-BChl e_G and isobutyl/ethyl [I,E]-BChl e_G eluted first. Homologs esterified with farnesol were ethyl/methyl [E,M]-BChl e_F, ethyl/ethyl [E,E]-BChl e_F, propyl/ethyl [P,E]-BChl e_F, isobutyl/ethyl [I,E]-BChl e_F. Trace amounts of the secondary homologs (mostly ethyl/ethyl-Bchl e_{HEN} and isobutyl/ethyl-BChl e_{HEN} esterified with hexadecenal) were also detected in some samples. Isr1, isorenieratene, Isr2, β-isorenieratene. Absorption spectra detected at 470 nm.



Supplementary Figure 2. Vertical distribution of BChle in the chemocline of the Black Sea at the eight main sampling locations.



Supplementary Figure 3. Survival of *Chlorobium* BS-1 during exposure to air in darkness. Time course of percentages of viable cells as determined by most probable number (MPN) enumerations relative to microscopic counts during a 2-week incubation experiment. Data points give average values, and vertical bars indicate 95% confidence limits. *Chlorobium* BS-1 was incubated under air (●), N_2 (▲), and under air, with 2.5 mM H_2S added to the culture (■). Data for the marine green sulfur bacterium *Chlorobium* strain GSB1 incubated under air (○) or N_2 (△) are given for comparison, since this strain is known to be highly resistant to prolonged exposure to air in the absence of light and sulfide (Beatty *et al.*, 2005). Asterisks indicate significant ($p < 0.05$) differences of MPN in BS-1 cultures incubated under air and N_2.



5.10 Supplementary tables

Supplementary Table 1. Sampling locations and sampling scheme of the present investigation during FS Meteor cruise M72-5. Only physicochemical parameters were determined at stations S1-S4 and S28-S33.

Station No.	Position		water depth (m)	Samples chemocline	*in situ* pumps (depth in m)	sediment
S1	42°20.0´N	32° 0.0´E	2235			
S2	42°30.0´N	32°30.0´E	2202			
S3	42°40.0´N	33° 0.0´E	2209			
S4	42°50.0´N	33°30.0´E	2212			
S5	43°22.0'N	32°38.0'E	2100	X	X (50,90,150)	X
S6	43°25.9'N	32°17.0'E	2025			X
S7	43°60.0'N	32° 1.8'E	1533	X		X
S8	44°37.7'N	32° 3.2'E	1063			X
S9	44°39.1'N	32° 1.0'E	1002			X
S10	44°44.5'N	32° 1.2'E	459	X	X (70,145,200,400)	X
S11	44°47.1'N	31°57.9'E	153			X
S12	44°49.8'N	31°55.6'E	75			X
S13	44°33.0'N	36°20.0'E	388	X		
S14	44°35.8'N	36°21.3'E	274			X
S15	44°37.2'N	36°21.9'E	155			X
S16	44°43.0'N	36°24.9'E	71			X
S17	44°41.1'N	36° 2.0'E	524			X
S18	44°34.2´N	36° 0.8'E	970			X
S20	43°57.3'N	35°38.5'E	2048	X		X
S21	42°45.0'N	37°30.0'E	2145	X	X (50,110,170,400)	X
S22	42°13.5'N	36°29.5'E	851			X
S23	41°36.3'N	37°26.4'E	1851	X		X
S24	41°28.6´N	37°11.7´E	208			
S25	42° 6.2'N	36°37.4'E	418			X
S27	42°23.8'N	36°16.0'E	1939	X		X
S28	42°57.0´N	34°30.0´E	2191			
S29	42°10.0´N	31°30.0´E	2150			
S30	42° 0.0´N	31° 0.0´E	2011			
S31	41°50.0´N	30°30.0´E	1949			
S32	41°45.0´N	30° 0.4´E	1883			
S33	41°35.0´N	29°25.0´E	1571			

Chapter 6

General Discussion

6.1 Freshwater *Sphingomonadaceae* as a model system for bacterial speciation

To analyze the microdiversity among closely related bacterial lineages an abundant and cultureable model system was needed. *Sphingomonadaceae* represent typical members of freshwater bacterioplankton communities (Gich *et al.*, 2005; Glöckner *et al.*, 2000; Zwart *et al.*, 2002) and aquatic *Sphingomonadaceae* have been successfully cultivated on low nutrient media (Page *et al.*, 2004; Pinhassi & Berman, 2003). Therefore *Sphingomonadaceae* were chosen as the target group.

6.1.1 Selection of a model phylotype

By a 16S rRNA gene library of pooled annual bacterioplankton samples *Sphingomonadaceae* accounted 27% of the alphaproteobacterial community in Walchensee. Two phylotypes, G1A and G7A, dominated the natural *Sphingomonadaceae* community (25 % and 42.5 % of the clones respectively). Both phylotypes were also found by denaturing gradient gelelectrophoresis (DGGE) of cDNA from reverse transcribed rRNA to be physiologically active *in situ* throughout most of the year, as indicated by the presence of their rRNA sequences in annual samples derived from the oligotrophic Walchensee and the neighboring mesotrophic Starnberger See. Furthermore 54 pure isolates of G1A and seven isolates from G7A could be recovered by targeted cultivation in diluted artificial freshwater medium. Consequently both abundant members of the natural environment are available in laboratory cultures.

Based on phylogenetic analysis, phylotype G1A forms a novel genus with 95.2% 16S rRNA gene sequence similarity to the closest related established *Sphingomonadaceae*, whereas phylotype G7A corresponds to *Sandarakinorhabdus limnophila* DSM 17366^T. One G1A isolate, strain G1A_585^T, was characterized in detail and was proposed as a new genus (*Sphingorhabdus planktonica* sp. nov., DSM 25081^T) based on the phylogenetically isolated position compared to the closest related genus *Sphingopyxis.* Furthermore differences in the composition of fatty acids, immobility and an exceptional low G+C content of 55.7 mol% distinguish the genus *Sphingorhabdus* from *Sphingopyxis.* Species within the genus

Sphingopyxis harbor DNA G+C contents between 62.3-69.2 mol%, with the exception of *Sphingopyxis flavimaris* DSM 16223^T that has a DNA G+C content of 58.0 mol%. *Sphingopyxis flavimaris* DSM 16223^T, *Sphingopyxis litoris* DSM 22379^T and *Sphingopyxis marina* DSM 22363^T form a branch that is distinct from all other *Sphingopyxis* species and cluster together with *Sphingorhabdus planktonica* DSM 25081^T based on 16S rRNA gene sequence analysis. The DNA G+C content from *S. litoris* and *S. marina* is unknown, thus further analysis is required to reveal whether this three *Sphingopyxis* species belong to the genus *Sphingorhabdus*.

The high number of 54 isolates recovered in this study, permitted population structure analysis within the phylotype G1A based on the laboratory cultures. Furthermore the abundance and annual activity of G1A *in situ* made us chosen this phylotype as target group to gain first insights into the processes that are involved in bacterial speciation and niche formation.

6.1.2 Microdiversity within the model phylotype based on ITS analysis

Among all isolates and sequences derived from the alphaproteobacterial library, members of the G1A-phylotype harbor identical 16S rRNA gene sequences. To resolve different subpopulations, the more variable internal transcribed spacer region (ITS1) was analyzed. Cultures and environmental sequences exhibited 5 and 4 different ITS1 sequences respectively. This diversity of cultured representatives surpasses the detected diversity of other studies. For example, intergenic spacer analysis among isolates of the marine Alphaproteobacterium *Candidatus Pelagibacter ubique* demonstrated the existence of three distinct ITS lineages, while one of these differed by a single base of the 16S rRNA gene sequence in addition (Rappé *et al.*, 2002). Among environmental sequences and those obtained from cultivated G1A representatives only two sequences were identical.

To increase the coverage, an Illumina sequencing approach of the same seasonal samples used for the cultivation and the clone library construction was employed and yielded 8,576 ITS1 sequences after a high quality check was performed. A strong quality control was needed, because small sequence differences had to be detected for the analysis of microdiverse clusters. In total 15 major and numerous rare sequence types were found in Starnberger See and Walchensee. The subsequent analysis of seasonal shifts was limited to the 15 detected major ITS1 sequence types, which surpassed an abundance threshold of 5% at least for one of the four seasonal sampling points. The G1A population in the mesotrophic Starnberger See comprised of eight major ITS-types that fluctuated in their annual abundance

pattern, while the G1A population in Walchensee was dominated by a single ITS1-type. The dynamic temporal patterns in Starnberger See may be caused by a larger number of ecological niches that were available to G1A in this environment and/or a more dynamic competition between the different ITS1-types of G1A compared to the Walchensee. In general the major ITS1-types exhibited distinct seasonal patterns suggesting that the corresponding *Sphingomonadaceae* lineages G1A may occupy different ecological niches.

6.1.3 Substrate utilization and niche partitioning

Substrate utilization patterns within and between the different cultivated ITS1-types of G1A were analyzed to identify potential ecological niches. Sixteen randomly chosen isolates belonging to the most abundant ITS1-type 4 were tested against 13 representatives of four other types (no. 2, 3, 5, 6). The substrate utilization patterns, as revealed by a BiOLOG assay, demonstrated that the variances of substrate utilization within the 16 isolates with identical ITS sequence (type 4) was higher than among the four other ITS types combined.

However, since strains of the same ITS1-type showed highly variable substrate utilization patterns, the potential mechanism of niche separation in G1A cannot be explained by substrate utilization alone and may be related to other traits. Potential mechanisms of niche adaptation in *Sphingomonadaceae,* especially in G1A, might include the degradation of refractory high-molecular-weight organic compounds (Balkwill *et al.*, 2006), resistance to UV radiation (Hörtnagl *et al.*, 2011), a planktonic or sessile life style (Balkwill *et al.*, 2006; Blom & Pernthaler, 2010; Schweitzer *et al.*, 2011), differences in the adaptation to different growth-limiting substrates (Eguchi *et al.*, 1996; Pinhassi & Hagström, 2000; Vancanneyt *et al.*, 2001), aerobic anoxygenic phototrophy (Gich & Overmann, 2006; Kim *et al.*, 2007), or a different susceptibility towards bacteriophage attack. The planktonic freshwater isolate *Sphingomonas sp.* strain B18 shows 99.2 % 16S rRNA sequence identity compared to G1A and is indeed sensitive to a broad spectrum of phages from different aquatic habitats (Wolf *et al.*, 2003). Future research along these lines is needed to elucidate the mechanisms that maintain the large variety of different ITS1 subpopulations for G1A as detected in the present study.

6.2 A uniform population of green sulfur bacteria from the Black Sea is adapted to extraordinary low light intensities

In contrast to the high microdiversity observed for freshwater *Sphingomonadaceae*, a homogeneous population of the green sulfur bacterium *Chlorobium* BS-1 was described in the Black Sea. Individual cells of this population exhibit identical 16S rRNA- and ITS1 sequences making *Chlorobium* BS-1 the largest known uniform population, while the adaptation mechanisms that lead to this unusual uniformity were unknown. *Chlorobium* BS-1 as an obligate phototrophic organism is adapted to an extreme ecological niche, the oxic-anoxic transition zone (chemocline) of Black Sea. Due to the dome-shaped structure of the chemocline, *Chlorobium* BS-1 is dwelling in 82-110 m depth below the surface at the center and at 150-180 m water depth at the periphery of the Black Sea (Manske *et al.*, 2005). Because of the low light intensities at these depths, *Chlorobium* BS-1 is adapted to the lowest light condition of any photosynthetic bacterium known so far. This part of the study focused on the underlying mechanisms that forced adaption to this extraordinary light limited niche, while investigating the factors that prevented microdiverse differentiations.

6.2.1 The ecological niche of *Chlorobium* BS-1

To elucidate the mechanisms that form the ecological niche for *Chlorobium* BS-1, water samples from different stations at the periphery and in the central basin of the Black Sea were collected during summer 2007. First, the ecological niche for BS-1 in the chemocline was analyzed more detailed.

ITS-RNA concentrations have previously been shown to reflect the activity of bacterial cells (Schmid *et al.*, 2001). Thus, the physiological state of BS-1 cells are reflected by the concentrations of their ITS1 transcripts, which were measured with RT-qPCR. ITS-RNA was only detected at the central stations (5 and 21), while no transcripts were present in the control sample from the 145 m deep chemocline close to the periphery (station 10).

Furthermore Evelyn Marschall and Jörg Overmann measured photosynthetic activity by Bacteriochlorophyll *e* concentrations and $H^{14}CO_3^-$ incorporation experiments. Highest concentrations of Bacteriochlorophyll *e* were determined at the two central stations, whereas the concentrations were lower at all other stations investigated. The light - dependent incorporation of $H^{14}CO_3^-$ was detected below 0.055 µmol quanta·m^{-2}·s^{-1} but above 0.0033 µmol quanta·m^{-2}·s^{-1} *in situ*. At the central stations light intensities of around 0.1 µmol quanta·m^{-2}·s^{-1} were measured and at all other stations below 0.055 µmol quanta·m^{-2}·s^{-1}. All measurements were conducted with samples derived from solar noon in summer,

corresponding to highest possible reachable light intensities in the Black Sea chemocline. Based on both measurements, BChl*e* concentrations and incorporation of $H^{14}CO_3^-$, photosynthetic growth of *Chlorobium* BS-1 in the natural population is possible in the center of the Black Sea basin, whereas at the periphery only very limited photosynthetic growth might be possible. Together with my measurements concerning the physiological state of the BS-1 cells, the ecological niche of the uniform population of *Chlorobium* BS-1 was assigned to the central shallow positions of the chemocline in the Black Sea.

6.2.2 Strategies of *Chlorobium* BS-1 to adapt to light limiting conditions in the natural environment

To adapt to the light limiting ecological niche in the Black Sea chemocline, *Chlorobium* BS-1 had to evolve specialized traits. At the most shallow position of the Black Sea the light intensity never exceeded 0.18 µmol quanta·m^{-2}·s^{-1}. At such low light intensities the BS-1 population *in situ* never exceeds light saturation, as previous laboratory measurements have shown (Manske *et al.*, 2005; Overmann *et al.*, 1992). Furthermore in winter the light intensities at the chemocline reached maxima of 0.0022 µmol quanta·m^{-2}·s^{-1} and thus were far below the photosynthesis threshold (Manske *et al.*, 2005).

To understand the survival and maintenance of the *Chlorobium* BS-1 population *in situ*, low light adapted laboratory cultures were incubated under different light conditions and ATP measurements were assessed. After 52 days of incubation at 0.0014 µmol Quanta m^{-2} s^{-1}, the ATP level dropped down to 74% of the initial value, which was still significantly higher than values of dark incubated cultures ($p < 0.01$). This result showes, that low-light adapted cells of BS-1 survive for a long period of time, while the light intensities corresponded to light intensities measured at the center in winter (≤ 0.0022 µmol quanta·m^{-2}·s^{-1}; (Manske *et al.*, 2005). Furthermore based on the ATP measurements, a maintenance energy value of ~1.6-4.9·10^{-15} kJ·$cell^{-1}$·d^{-1} was calculated. Thus, the maintenance energy determined for *Chlorobium* BS-1 represents the lowest value detected for any bacterial culture. These findings demonstrate that BS-1 is well adapted to long-term survival under extreme low light conditions in the Black Sea's chemocline, even in winter were no photosynthesis is possible.

One further explanation for the low light adaptation of *Chlorobium* BS-1 can be derived from Evelyn Marschalls and Jörg Overmanns pigment analyses of natural samples. BS-1 cells from deeper locations of the chemocline contained Bacteriochlorophyll *e* homologs with longer aliphatic side chains than light saturated incubated laboratory cultures. Such side chains could lead to a red shift of the absorption maximum, that has been hypothesized to

enhance the channeling of excitation energy and to increase the energy transfer efficiency from the chlorosomes towards the reaction (Borrego & Garcia-Gil, 1995). In addition, this shift has previously been documented for laboratory cultures of *Chlorobium* BS-1 after transfer to lower light intensities (Manske *et al.*, 2005).

In conclusion, the ecological niche of *Chlorobium* BS-1 was found to be the chemocline of the central basin of the Black Sea. The uniformity of the population seems to be caused by specific requirements related to low-light adaptation, which counteract speciation. Thus, no microdiversity could eventually arise in this extreme example of adaptation.

6.3 References

Balkwill, D. L., Frederickson, J. K. & Fromine, M. F. (2006). *Sphingomonas* and related genera. *Prokaryotes* **7**, 605-629.

Blom, J. F. & Pernthaler, J. (2010). Antibiotic effects of three strains of chrysophytes (*Ochromonas*, *Poterioochromonas*) on freshwater bacterial isolates. *FEMS Microbiol Ecol* **71**, 281-290.

Borrego, C. M. & Garcia-Gil, L. J. (1995). Rearrangement of light harvesting bacteriochlorophyll homologues as a response of green sulfur bacteria to low light intensities. *Photosynthesis research* **45**, 21-30.

Eguchi, M., Nishikawa, T., MacDonald, K., Cavicchioli, R., Gottschal, J. & Kjelleberg, S. (1996). Responses to stress and nutrient availability by the marine ultrabacterium *Sphingomonas* sp. strain RB2256. *Appl Environ Microbiol* **62**, 1287-1294.

Gich, F. & Overmann, J. (2006). *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a novel bacteriochlorophyll a-containing, obligately aerobic bacterium isolated from freshwater lakes. *Int J Syst Evol Microbiol* **56**, 847-854.

Gich, F., Schubert, K., Bruns, A., Hoffelner, H. & Overmann, J. (2005). Specific detection, isolation and characterization of selected, previously uncultured members of freshwater bacterioplankton. *Appl Environ Microbiol* **71**, 5908-5919.

Glöckner, F. O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R. (2000). Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. *Appl Environ Microbiol* **66**, 5053-5065.

Hörtnagl, P., Pérez, M. T. & Sommaruga, R. (2011). Contrasting effects of ultraviolet radiation on the growth efficiency of freshwater bacteria. *Aquat Ecol* **45**, 125-136.

Kim, M. K., Schubert, K., Im, W.-T., Kim, K.-H., Lee, S.-T. & Overmann, J. (2007). *Sphingomonas kaistensis* sp. nov., a novel alphaproteobacterium containing *pufLM* genes. *Int J Syst Evol Microbiol* **57**, 1527-1534.

Manske, A. K., Glaeser, J., Kuypers, M. M. & Overmann, J. (2005). Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. *Appl Environ Microbiol* **71**, 8049-8060.

Overmann, J., Cypionka, H. & Pfennig, N. (1992). An extremely low-light adapted phototrophic sulfur bacterium from the Black Sea. *Limnol Oceanogr* **37**, 150-155.

Page, K. A., Connon, S. A. & Giovannoni, S. J. (2004). Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. *Appl Environ Microbiol* **70**, 6542-6550.

Pinhassi, J. & Hagström, Å. (2000). Seasonal succession in marine bacterioplankton. *Aquat Microb Ecol* **21**, 245-256.

Pinhassi, J. & Berman, T. (2003). Differential growth response of colony-forming α- and γ-*Proteobacteria* in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat. *Appl Environ Microbiol* **69**, 199-211.

Rappé, M. S., Connon, S. A., Vergin, K. L. & Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630-633.

Schmid, M., Schmitz-Esser, S., Jetten, M. & Wagner, M. (2001). 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environ microbiol* **3**, 450-459.

Schweitzer, B., Huber, I., Amann, R., Ludwig, W. & Simon, M. (2011). *α- and β-Proteobacteria* control the consumption and release of amino acids on lake snow aggregates. *Appl Environ Microbiol* **67**, 632-645.

Vancanneyt, M., Schut, F., Snauwaert, C., Goris, J., Swings, J. & Gottschal, J. C. (2001). *Sphingomonas alaskensis* sp. nov., a dominant bacterium from a marine oligotrophic environment. *Int J Syst Evol Microbiol* **51**, 73-79.

Wolf, A., Wiese, J., Jost, G. & Witzel, K. P. (2003). Wide geographic distribution of bacteriophages that lyse the same indigenous freshwater isolate (*Sphingomonas sp.* strain B18). *Appl Environ Microbiol* **69**, 2395-2398.

Zwart, G., Crump, B. C., Kamst-Van Agterveld, M. P., Hagen, F. & Han, S. (2002). Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28**, 141-155.

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